



## Supplementary Materials for

### **Architecture of the fungal nuclear pore inner ring complex**

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**Other Supporting Online Material for this manuscript includes the following:**  
(available at [www.sciencemag.org/cgi/content/full/science.aac9176/DC1](http://www.sciencemag.org/cgi/content/full/science.aac9176/DC1))

Movies S1 to S4

## Materials and Methods

### Bacterial expression constructs

DNA fragments were amplified by PCR using *C. thermophilum* and *H. sapiens* cDNA. SUMO (small ubiquitin-like modifier) tagged proteins were cloned into a modified pET28a vector or a modified pET-MCN vector containing an N-terminal hexahistidine tag followed by a SUMO tag using BamHI and NotI restriction sites (42, 43). Hexahistidine tagged proteins were cloned into a modified pET28a vector containing a N-terminal hexahistidine tag followed by a PreScission protease cleavage site using NdeI and NotI restriction sites (44). GST tagged proteins were cloned into pGEX-6P1 using the BamHI and NotI restriction sites. Additionally, DNA coding for Nsp1 (residues 467-674) and Nup57 (residues 77-319) or Nup57 (residues 146-319) were cloned sequentially into pETDuet1 (Novagen) for expression of untagged protein. Nsp1 was cloned into the first multiple cloning site using NcoI and NotI restriction sites while Nup57 was cloned into the second multiple cloning site using NdeI and XhoI restriction sites. The selected synthetic antibody (sAB) fragments of sAB-158 and sAB-160 were cloned into the pSFV4 vector (Peter Loppnau, Structural Genomics Consortium, University of Toronto) using the restriction sites NcoI and Sall, and subsequently digested using Sall and BsaI and re-ligated to obtain the C-terminal hexahistidine tag. Mutants were generated by QuikChange mutagenesis and confirmed by DNA sequencing. Details of expression constructs are shown in [Table S5](#).

### Protein expression and purification

Proteins were expressed in *E. coli* BL21-CodonPlus(DE3)-RIL cells (Stratagene) in Luria-Bertani media and induced at an OD 600 of 0.8 with 0.5 mM IPTG. For details of the expression times and temperatures see [Table S5](#). Seleno-L-methionine (SeMet) labeled proteins were expressed by a methionine pathway inhibition protocol, as previously described (45). Cells were harvested by centrifugation and re-suspended in a buffer containing 20 mM TRIS (pH 8.0), 500 mM NaCl, 5 mM 2-mercaptoethanol ( $\beta$ -ME), supplemented with complete EDTA-free protease inhibitor cocktail (Roche). For purification, the cell suspensions of all proteins and protein complexes were supplemented with 1 mg DNase I (Roche) and lysed using a cell disruptor (Avestin). Cell lysates were cleared by centrifugation at 30,000 x g for 1 hour. The supernatants were filtered through a 0.45- $\mu$ m filter (Millipore) and purified using standard chromatography methods; for details see [Table S6](#). Proteins were concentrated to ~10-20 mg/ml for biochemical interaction experiments, complex reconstitution, and crystallization.

**Purification of Nup192•Nic96<sup>R2-SOL</sup>.** Nup192 and GST tagged Nic96<sup>R2-SOL</sup> were expressed individually. After harvesting cells, were re-suspended in 50 mM TRIS (pH 8.0), 500 mM NaCl, 5 mM  $\beta$ -ME, 10 % (v/v) glycerol supplemented with complete EDTA-free protease inhibitor cocktail (Roche), 2  $\mu$ M bovine lung aprotinin (Sigma) and 1 mM phenylmethanesulfonyl fluoride (PMSF; Sigma). Prior to lysis, cell suspensions of GST-Nic96<sup>R2-SOL</sup> and Nup192 were mixed in a 1:2 ratio. Clarified lysate was loaded onto a Ni-NTA column equilibrated in a buffer containing 25 mM TRIS (pH 8.0), 500 mM NaCl, 5 mM  $\beta$ -ME, 20 mM imidazole, and 5 % (v/v) glycerol and eluted with an imidazole gradient. Peak fractions were pooled and loaded onto a Glutathione Sepharose 4 Fast Flow (GE Healthcare) column and eluted using a glutathione gradient. Peak fractions were pooled and dialyzed overnight against a buffer containing 20 mM TRIS (pH 8.0), 150 mM NaCl, 5 mM DTT, and 5 % (v/v) glycerol in the presence of PreScission protease. The protein was concentrated and further purified over a HiLoad Superdex 200 16/60 PG column equilibrated in a buffer containing 20 mM TRIS (pH 8.0), 150 mM NaCl, and 5 mM DTT. Peak fractions were pooled and concentrated to ~20 mg/ml for biochemical interaction experiments.

**Purification of CNT•Nic96.** Cells containing CNT and Nic96 were grown individually and re-suspended in 50 mM TRIS (pH 8.0), 500 mM NaCl, 4 mM  $\beta$ -ME, 10 % (v/v) glycerol supplemented with complete EDTA-free protease inhibitor cocktail (Roche), 2  $\mu$ M bovine lung aprotinin (Sigma) and 1 mM phenylmethanesulfonyl fluoride (PMSF; Sigma). Prior to lysis, cell suspension containing CNT and Nic96 were mixed in a 3:1 ratio. Clarified lysate was loaded onto a Ni-NTA column equilibrated in a buffer containing 25 mM TRIS (pH 8.0), 500 mM NaCl, 5 mM  $\beta$ -ME, 20 mM imidazole, and 5 % (v/v) glycerol and eluted with an imidazole gradient. Peak fractions were pooled and desalted using a HiPrep

26/20 Desalting (GE Healthcare) column equilibrated in a buffer containing 20 mM TRIS (pH 8.0), 150 mM NaCl, 5 mM DTT, and 5 % (v/v) glycerol in the presence of ULP1 protease. After SUMO cleavage, the protein was loaded onto a MonoQ 5/50 GL (GE Healthcare) column equilibrated in 20 mM TRIS (pH 8.0), 150 mM NaCl, 5 mM DTT, and 5 % (v/v) glycerol and eluted with a sodium chloride gradient. Protein-containing fractions were pooled and concentrated to ~5 mg/ml for biochemical interaction experiments.

**Purification of sABs.** The expression of sABs was induced at an OD 600 of 0.9 with 0.25 mM IPTG and grown at 37 °C for 3 hours. The lysate was incubated at 65 °C for 30 minutes and then cooled on ice for 15 minutes before centrifugation at 30,000 x g for 1 hour. The supernatant was filtered through a 0.45-µm filter (Millipore) and loaded onto a Ni-NTA column equilibrated in 20 mM TRIS (pH 8.0), 500 mM NaCl, and 20 mM imidazole. Protein was eluted with a linear gradient of 20 mM TRIS (pH 8.0), 500 mM NaCl, and 500 mM imidazole. Protein-containing fractions from the Ni-NTA affinity purification were pooled and loaded onto a 5 ml HiTrap MabSelect SuRe (GE Healthcare) column equilibrated in a buffer containing 20 mM TRIS (pH 8.0) and 500 mM NaCl and eluted using a linear gradient of an elution buffer containing 0.1 M acetic acid. The eluted fractions were dialyzed against a buffer containing 20 mM TRIS (pH 8.0) and 100 mM NaCl and concentrated to ~50 mg/ml for biochemical interaction experiments, complex reconstitution, and crystallization.

**Purification of CNT•Nic96<sup>R1</sup>.** Purified CNT was mixed with 2-fold molar excess of purified SUMO-Nic96<sup>R1</sup> and incubated on ice for 30 minutes. The mixture was then loaded onto a HiLoad Superdex 200 16/60 PG column equilibrated in buffer containing 20 mM TRIS (pH 8.0), 100 mM NaCl, and 5 mM DTT. To obtain untagged CNT•SUMO-Nic96<sup>R1</sup>, the proteins were mixed in the presence of ULP1 protease. Fractions containing either CNT•SUMO-Nic96<sup>R1</sup> or untagged CNT•Nic96<sup>R1</sup> were pooled and concentrated to ~30 mg/ml for biochemical studies.

**Purification of CNT•Nic96<sup>R1</sup>•sAB-158.** Purified CNT was mixed with 2-fold molar excess of purified SUMO-Nic96<sup>R1</sup>, 2-fold molar excess of purified sAB-158, and ULP1 protease, followed by incubation on ice for 30 minutes. The mixture was then loaded onto a Superdex 200 10/300 GL column equilibrated in buffer containing 20 mM TRIS (pH 8.0), 100 mM NaCl, and 5 mM DTT. Fractions containing CNT•Nic96<sup>R1</sup>•sAB-158 were pooled and concentrated to ~50 mg/ml for biochemical studies and crystallization.

**Purification of Nup82<sup>NTD</sup>•Nup159<sup>T</sup>•Nup145N<sup>APD</sup>.** Purified Nup82<sup>NTD</sup>•Nup159<sup>T</sup> was mixed with a 1.2-fold molar excess of Nup145N<sup>APD</sup> and incubated on ice for 30 minutes. The mixture was then loaded onto a Superdex 200 16/60 PG column equilibrated in buffer containing 20 mM TRIS (pH 8.0), 100 mM NaCl, and 5 mM DTT. Peak fractions containing a stoichiometric Nup82<sup>NTD</sup>•Nup159<sup>T</sup>•Nup145N<sup>APD</sup> hetero-trimer were pooled and concentrated to ~30 mg/ml for biochemical studies and crystallization.

**Reconstitution of hsNup49<sup>CCS2+3\*</sup>•hsNup57<sup>CCS3\*</sup> complexes.** Purified hsNup49<sup>CCS2+3\*</sup> was mixed with an equimolar amount of hsNup57<sup>CCS3\*</sup> and incubated on ice for 30 minutes. The mixture was then loaded onto a HiLoad Superdex 200 16/60 PG column equilibrated in buffer containing 20 mM TRIS (pH 8.0), 100 mM NaCl, and 5 mM DTT. Peak fractions containing a stoichiometric hsNup49<sup>CCS2+3\*</sup>•hsNup57<sup>CCS3\*</sup> complex were pooled and concentrated to ~20 mg/ml for crystallization.

**Reconstitution of CNT•Nic96•Nup192•Nup53.** The hetero-nonameric IRC protomer including the CFC proteins Nup82<sup>NTD</sup> and Nup159<sup>T</sup> was reconstituted as follows. A Nup192•Nup53 complex was formed by mixing purified Nup192 and Nup53 with a 1.2 fold molar excess of Nup53, incubated on ice for 30 minutes and purified over a HiLoad Superdex 200 16/60 PG column equilibrated in a buffer containing 20 mM TRIS (pH 8.0), 100 mM NaCl and 5 mM DTT. The Nup192•Nup53 dimer was subsequently mixed with a 1.2-fold molar excess of CNT•Nic96, incubated for 30 minutes on ice and further purified over a HiLoad Superdex 200 16/60 PG gel filtration column equilibrated in 20 mM TRIS (pH 8.0), 100 mM NaCl and 5 mM DTT. Complex-containing fractions were pooled and concentrated to ~8 mg/ml for biochemical studies.

**Reconstitution of CNT•Nic96•Nup192•Nup53•Nup145N•Nup82<sup>NTD</sup>•Nup159<sup>T</sup>.** To reconstitute CNT•Nic96•Nup192•Nup53•Nup145N•Nup82•Nup159, 1 mg of purified CNT•Nic96•Nup192•Nup53 was mixed with 0.8 mg of purified Nup82<sup>NTD</sup>•Nup159<sup>T</sup>•Nup145N, incubated for 30 minutes on ice, and

purified over a Superose 6 10/300 GL column equilibrated in 20 mM TRIS (pH 8.0), 100 mM NaCl and 5 mM DTT.

### **Generation of synthetic antibodies and monobodies**

The selection of synthetic binders was performed using avi-tagged CNT, CNT•Nic96<sup>R1</sup>, and CNT•Nic96<sup>139-201</sup>, containing Nup49 with an N-terminal avi-tag. Biotinylation was carried out using 40  $\mu$ M protein in a buffer containing 50 mM BICINE (pH 8.3), 100 mM biotin, 10 mM ATP, 10 mM magnesium acetate, and 30  $\mu$ g biotin ligase (BirA) at 30 °C for 2 hours in a total volume of 2 ml. After labeling, protein was buffer exchanged using a 5 ml HiTrap Desalting (GE Healthcare) column equilibrated with a buffer containing 20 mM TRIS (pH 8.0), 100 mM NaCl, and 5 mM DTT and purified again using a HiLoad Superdex 200 16/60 PG column equilibrated in the same buffer. The extent of biotinylation and efficiency of capture were tested by incubating 10  $\mu$ g protein with 50  $\mu$ l of Streptavidin MagneSphere Paramagnetic Particles (Promega), followed by a single washing step with 50  $\mu$ l buffer, containing 20 mM TRIS (pH 8.0), 100 mM NaCl, and 5 mM DTT, and resolving the bound proteins by SDS-PAGE and visualizing by Coomassie Brilliant Blue staining.

For sAB identification, four rounds of competitive selection were performed using 100 nM (round 1), 50 nM (round 2), 10 nM (round 3), and 10 nM (round 4) biotinylated CNT, CNT•Nic96<sup>R1</sup>, or CNT•Nic96<sup>139-201</sup> and a phage display library according to previously published protocols (46, 47). After successful selection, the specificity of candidate sABs was tested against the biotinylated CNT, CNT•Nic96<sup>R1</sup>, and CNT•Nic96<sup>139-201</sup> using a single point competitive ELISA assay (46). Only sequence-unique sABs with the desired binding properties were chosen for further biochemical characterization. Monobody libraries were screened with a similar protocol and affinities determined by yeast display, as previously described (48, 49).

To evaluate the binding affinity and specificity of the selected sABs and MBs, 1.5-fold molar excess of sAB or MB was incubated with CNT, CNT•Nic96<sup>R1</sup>, or CNT•Nic96<sup>139-201</sup> and loaded onto a MonoQ 5/50 GL column equilibrated in a buffer containing 20 mM TRIS (pH 8.0), 100 mM NaCl, and 5 mM DTT and eluted using a NaCl gradient. Interacting sABs or MBs eluted with the CNT, CNT•Nic96<sup>R1</sup>, or CNT•Nic96<sup>139-201</sup>, whereas non-interacting sABs or MBs eluted prior to the gradient step. The screened sABs or MBs formed stable complexes with CNT, CNT•Nic96<sup>R1</sup>, and CNT•Nic96<sup>139-201</sup>, but failed to differentiate between them.

### **Multi-angle light scattering coupled to analytical size-exclusion chromatography**

Purified proteins and complex formation were characterized by inline multi-angle light scattering following separation on Superdex 200 10/300 GL or Superose 6 10/300 GL columns (50). All proteins and complexes were analyzed in a buffer containing 20 mM TRIS (pH 8.0), 100 mM NaCl and 5 mM DTT, except those involving Nup192<sup>TAIL</sup>, which were carried out in a buffer containing 20 mM TRIS (pH 8.0), 200 mM NaCl and 5 mM DTT. The chromatography system was connected in series with an 18-angle light scattering detector (DAWN HELEOS II, Wyatt Technology), a dynamic light scattering detector (DynaPro Nanostar, Wyatt Technology), and a refractive index detector (Optilab t-rEX, Wyatt Technology). Data were collected every 1 second at a flow rate of 0.5 ml/min at 25 °C and analyzed using ASTRA 6 software, yielding molar mass and mass distribution (polydispersity) of the samples. For interaction studies, proteins were mixed and incubated on ice for 30 minutes prior to being applied to the gel filtration column. Protein containing fractions were analyzed by SDS-PAGE followed by Coomassie brilliant blue staining.

### **Thermal stability assay**

Purified CNT (10  $\mu$ g) was incubated in a total volume of 50  $\mu$ l for 30 minutes at 40-61 °C, increased in 3.5 °C increments. Soluble and pellet fractions were separated by centrifugation at 30,000 x g for 35 minutes at 4 °C, resolved by SDS-PAGE, and visualized by Coomassie brilliant blue staining.

### **GST pull-down protein-protein interaction analysis**

Bait protein (5  $\mu$ g, purified GST-Nic96<sup>R1</sup> or GST) was incubated for 30 minutes with an equimolar amount of target proteins in a buffer containing 20 mM TRIS (pH 8.0), 100 mM NaCl, 4 mM DTT. The



mixtures were then incubated for 30 minutes with 20  $\mu$ l pre-washed Glutathione Sepharose 4B resin (GE Healthcare). The beads were collected by centrifugation at 500 x *g* for 2 minutes and washed five times with 200  $\mu$ l binding buffer. Bound proteins were released from the resin by addition of 0.5  $\mu$ g PreScission protease and incubation for 20 minutes at room temperature. Samples from the load, PreScission eluted supernatant, and post-elution beads were analyzed by SDS-PAGE and visualized by Coomassie brilliant blue staining. Because Nup49 and Nup57 were poorly behaved when expressed individually, they were not tested in isolation.

### **Yeast strains, media and miscellaneous genetic methods**

Preparation of all media (yeast peptone dextrose, YPD; synthetic dextrose complete, SDC), LiAc-mediated yeast transformation and genetic manipulation was carried out according to standard protocols. For details of the yeast expression constructs and haploid *S. cerevisiae* strains see [Tables S7 and S8](#). To generate the listed shuffle strains the respective essential genes were replaced with either the *HIS3* or the *natNT2* cassette by homologous recombination, as previously described (51, 52). Due to the lethality of the deletions, the indicated parental strain was complemented with a pRS416 construct carrying the respective full-length *S. cerevisiae* gene with an N-terminal mCherry tag under the control of the *P<sub>NOP1</sub>* promoter prior to chromosomal integration. Subsequently, pRS415-eGFP or pRS415-mCherry constructs carrying the various Nup variants were introduced. The transformants were selected twice on SDC-leucine (Leu) plates followed by plating twice on SDC+5-fluoroorotic acid (5-FOA) to ensure the loss of the pRS416-mCherry construct prior to analysis. The *NUP192* shuffle constructs and the *nup49-313* mutant (EVPIP) were described previously (8, 37).

### **Yeast cell growth analyses and fluorescence microscopic assays**

For viability and growth analysis, *S. cerevisiae* strains carrying eGFP-tagged variants of the respective *scNUP* were grown at 30 °C. Ten-fold dilution series were generated, of which 20  $\mu$ l were spotted on SDC-Leu and SDC+5-FOA plates and grown at 23 °C for 5 days. For growth analysis of the shuffled strains, 7.5  $\mu$ l of the dilution series were spotted on YPD plates and grown at 23 °C, 30 °C, and 37 °C for 2-4 days. For *scNup57* co-localization analysis, cells with a genomic, C-terminal GFP-tagged *scNUP57* and pRS415-mCherry constructs carrying the various *NUP* variants in the respective knockout background were grown at 30 °C to mid log phase and afterwards shifted for 6 hours to 37 °C. The large ribosomal subunit *scRpl25* reporter assay was performed as described previously (53). The pRS411 (for *scNUP192* and *scNIC96* mutant analysis) or the pRS413 (for *scNUP49* mutant analysis) vectors carrying mCherry-tagged *scRPL25* were co-transformed with the respective pRS415-eGFP variant plasmids (see above) into the indicated shuffle strain. The transformants were selected twice on SDC-LEU-MET or SDC-LEU-HIS plates followed by plating twice on SDC-MET+5-FOA or SDC-HIS+5-FOA to ensure the loss of the pRS416-mCherry construct. For analysis, cells were grown in selective SDC-MET or SDC-HIS medium at 30 °C to mid-log phase. For fluorescence microscopy, live cells were washed once with water, re-suspended and analyzed using a Carl Zeiss Observer Z.1 equipped with a Hamamatsu camera C10600 Orca-R2. The statistical analysis was carried out using three sets of independent images with at least 500 cells each for every analyzed sample. Error bars represent standard deviation.

### **FISH mRNA Export Assay**

Liquid cultures of deletion yeast strains were grown at 30 °C (23 °C for the *nic96 $\Delta$ /Nup57-GFP* strain) in YPD media to an OD 600 of 0.4 and subsequently shifted to 37 °C for 4 hours prior to fixation in formaldehyde. These cells were then analyzed by FISH using an Alexa647-labeled 50-mer oligo(dT) probe as previously described (54, 55). The statistical analysis was carried out using three sets of independent images with at least 500 cells each for every analyzed sample. Error bars represent standard deviation.

### **Crystallization and structure determination**

Crystals of Nup192<sup>TAIL</sup>, Nup188<sup>TAIL</sup>, hsNup49<sup>CCS3\*</sup>, hsNup57<sup>CCS3\*</sup>, Nup57<sup>CCS3\*</sup>, hsNup49<sup>CCS2+3\*</sup>•hsNup57<sup>CCS3\*</sup> with 2:4 and 2:2 stoichiometry, Nup82<sup>NTD</sup>•Nup159T•Nup145N<sup>APD</sup>, and

CNT•Nic96<sup>R1</sup>•sAB-158 were grown at 21 °C in hanging drops containing 1 µl of protein and 1 µl of a reservoir solution. SeMet-labeled crystals were grown under similar conditions. For CNT•Nic96<sup>R1</sup>•sAB-158, native crystals were derivatized with *p*-chloromercuribenzoic acid (PCMB), ethyl mercury thiosalicylate (EMTS), potassium hexachloroosmate (K<sub>2</sub>OsCl<sub>6</sub>), and potassium osmate (K<sub>2</sub>OsO<sub>4</sub>). Crystals were cryoprotected by supplementing the drop with cryo protectant and flash-frozen in liquid nitrogen. For details of the crystallization and cryo protection see [Table S9](#). X-ray diffraction data were collected at 100 K at beamline 8.2.2 at the Advanced Light Source (ALS), Lawrence Berkley National Laboratory (LBNL), beamline BL12-2 at the Stanford Synchrotron Radiation Source (SSRL), and beamline GM/CA-CAT 23ID-D at the Advanced Photon Source (APS). The X-ray diffraction data were processed using the HKL2000 and XDS (56, 57).

Initial phases for all structures were calculated in PHASER using single anomalous dispersion (SAD) X-ray diffraction data obtained from SeMet derivatives (58). Solvent flattening and NCS averaging were performed in RESOLVE, yielding improved phases and experimental maps of excellent quality. In the case of CNT•Nic96<sup>R1</sup>•sAB-158, the best experimental map was obtained using SAD X-ray diffraction data obtained from the K<sub>2</sub>OsCl<sub>6</sub> derivative and inclusion of the data in the highest resolution shell was beneficial for its quality. Iterative rounds of model building and refinement were performed with COOT and PHENIX (59, 60). In the cases of Nup188<sup>TAIL</sup> and *hs*Nup49<sup>CCS2+3\*</sup>, the refinements did not converge in higher symmetry space groups and were therefore carried out in P1. The unambiguous assignment of the CNT•Nic96<sup>R1</sup>•sAB-158 sequence register was aided by anomalous difference Fourier map calculations using the derivatives. Subsequently, the assigned sequence register was verified by methionine mutants yielding 22 additional selenium sites (Nup49: I263M, H276M, L31M, A307M, L332M, E438M; Nup57: I82M, H109M, L113M, E127M, I138M, L198M, I233M, L253M, V304M; Nsp1: L527M, L548M, A628M; and Nic96<sup>R1</sup>: L140M, L158M, L161M, L178M). In total 78 heavy atom sites were identified in anomalous difference Fourier maps ([fig. S29](#)).

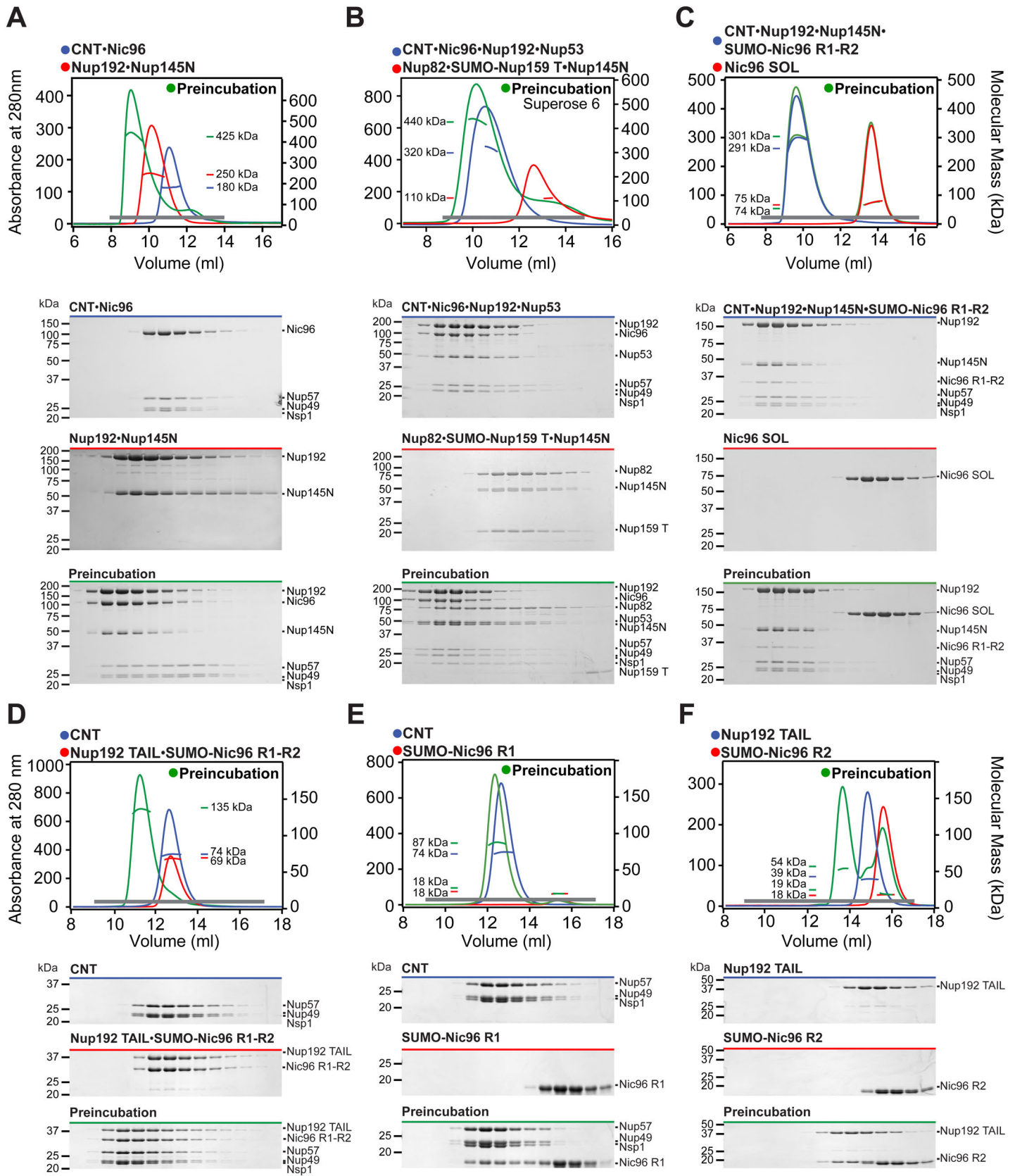
For all structures, the final models possess excellent  $R_{\text{work}}$  and  $R_{\text{free}}$  values and the assessment of their stereochemical qualities with MolProbity revealed no Ramachandran outliers (61). For details of the data collection and refinement statistics see [Tables S2 to S4](#).

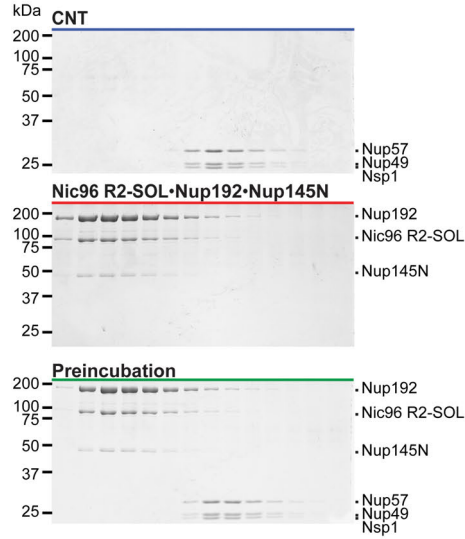
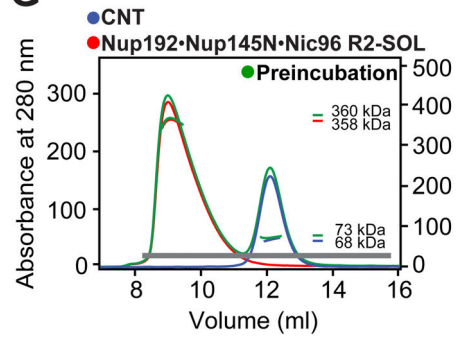
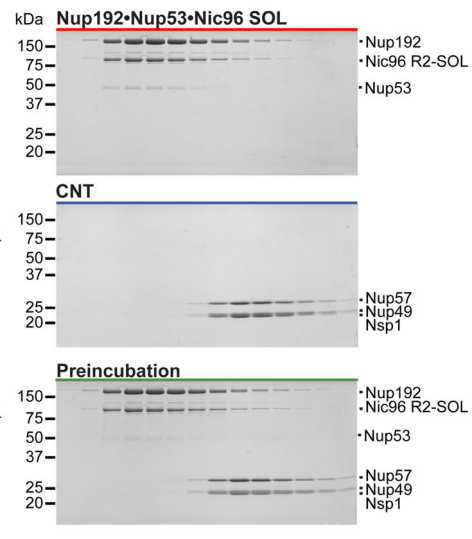
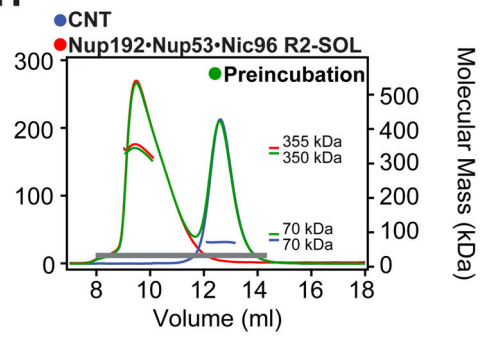
### Illustrations and figures

Gel filtration profiles and MALS graphs were generated in IGOR (WaveMetrics) and assembled in Adobe Illustrator. Sequence alignments were generated using ClustalX (62) and colored with ALSCRIPT (63). Electrostatic potentials were calculated with APBS (Adaptive Poisson-Blotzmann Solver) software (64). Structure figures were generated with PyMOL ([www.pymol.org](http://www.pymol.org)).

### Coordinates and Structure Factors

The coordinates and structure factors have been deposited with the Protein Data Bank with accession codes 5CWV (Nup192<sup>TAIL</sup>), 5CWU (Nup188<sup>TAIL</sup>), 4JQ5 (*hs*Nup49<sup>CCS2+3\*</sup>), 4JNV and 4JNU (*hs*Nup57<sup>CCS3\*</sup>), 5CWT (Nup57<sup>CCS3\*</sup>), 4JO7 (*hs*Nup49<sup>CCS2+3\*</sup>•*hs*Nup57<sup>CCS3\*</sup>, 2:2 stoichiometry), 4JO9 (*hs*Nup49<sup>CCS2+3\*</sup>•*hs*Nup57<sup>CCS3\*</sup>, 2:4 stoichiometry), 5CWW (Nup82<sup>NTD</sup>•Nup159<sup>T</sup>•Nup145N<sup>APD</sup>) and 5CWS (CNT•Nic96<sup>R1</sup>•sAB-158).

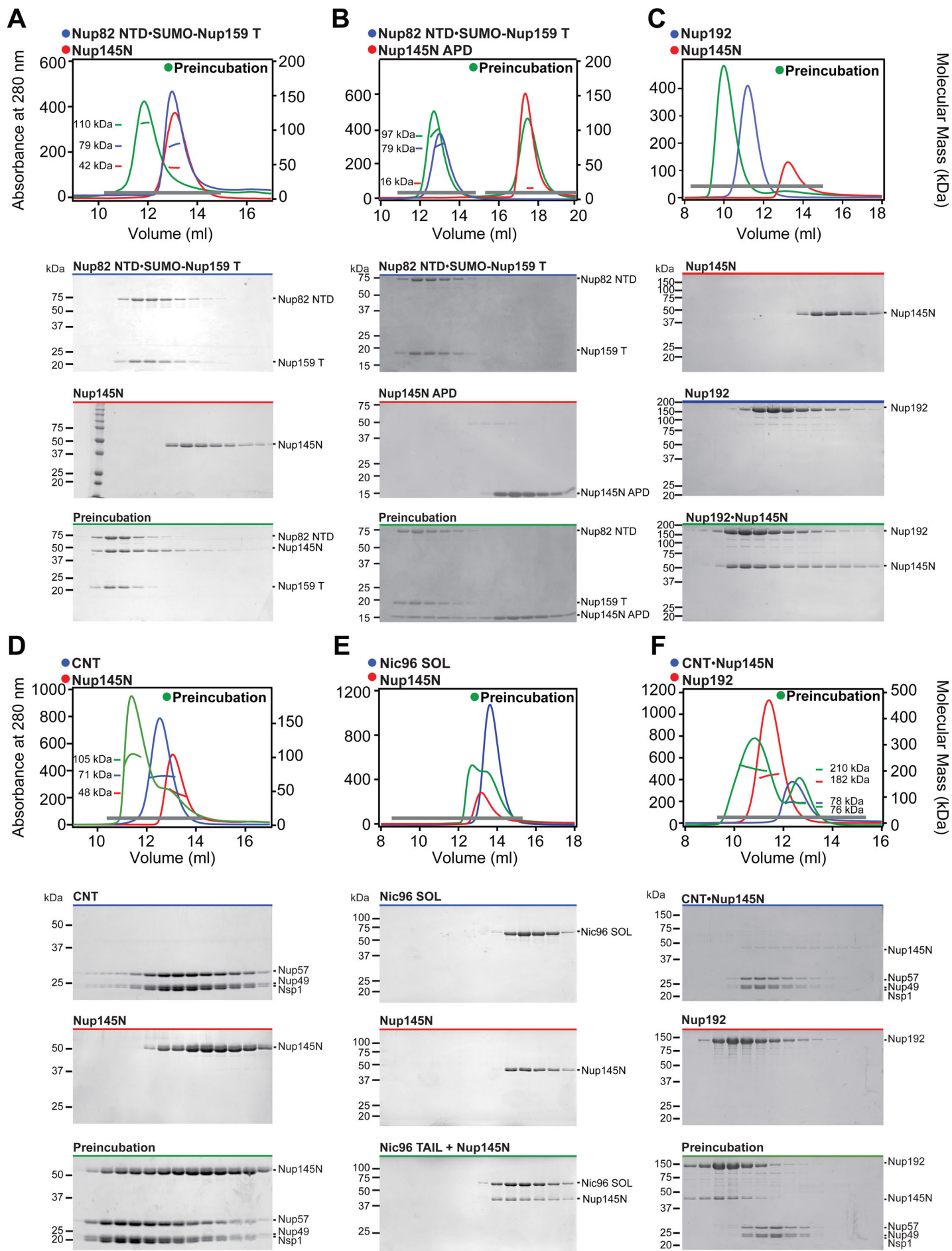


**G****H**

**Fig. S1.**

**Reconstitution and dissection of the IRC.** (A to H) SEC-MALS and SDS-PAGE analysis corresponding to [Figure 1](#). SEC-MALS profiles of nucleoporins or nup complexes are shown individually (red and blue) and after their preincubation (green). Measured molecular masses are indicated for the peak fractions. Gray bars indicate fractions that were resolved on SDS-PAGE gels and visualized by Coomassie staining.

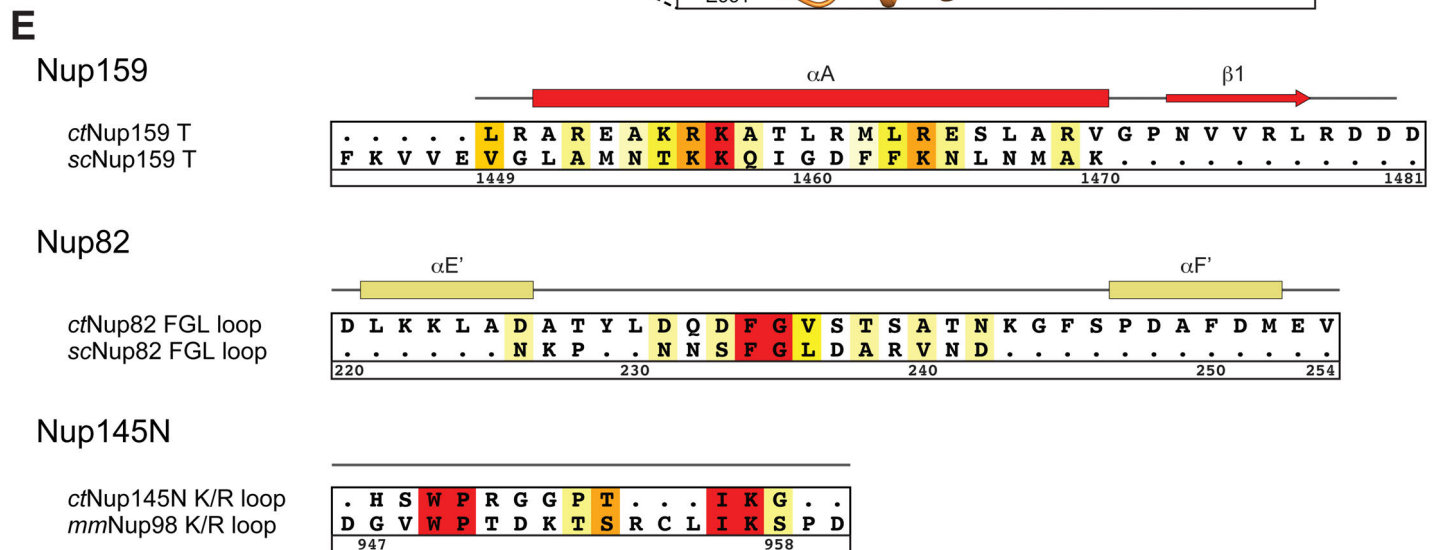
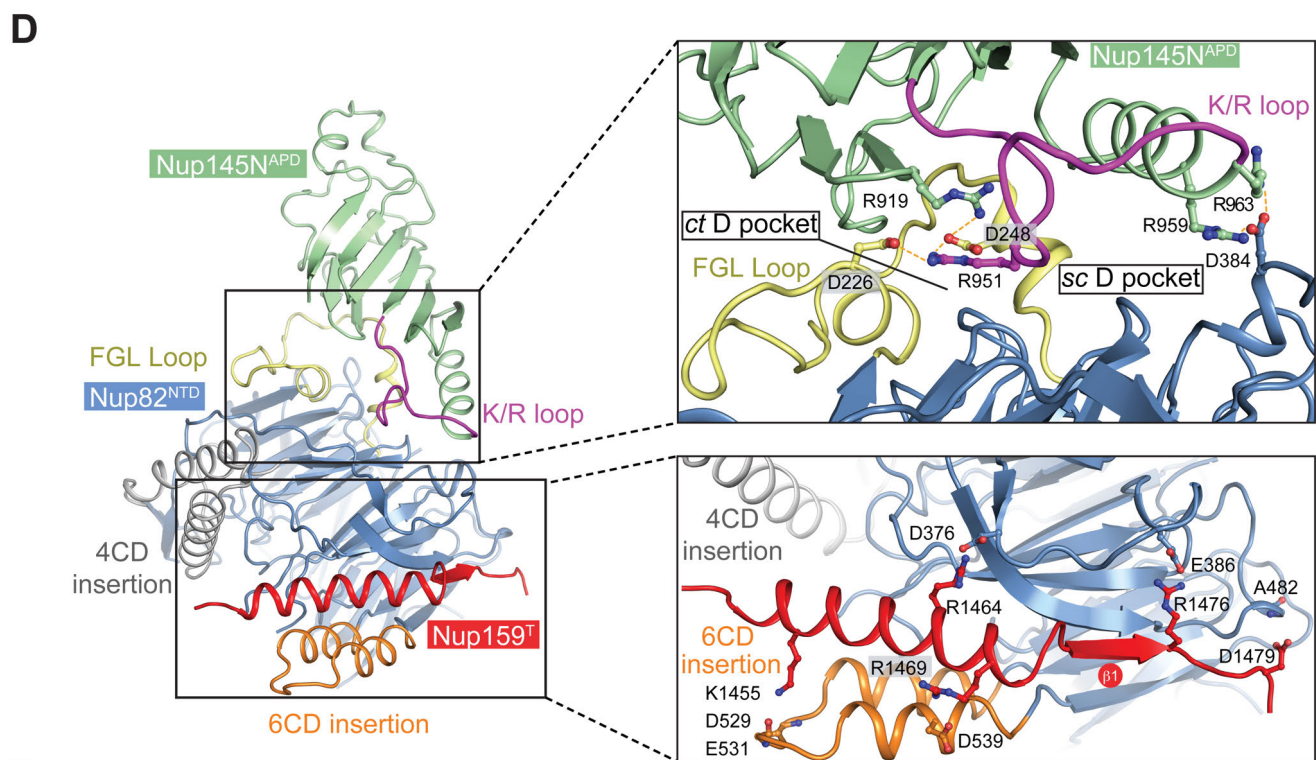
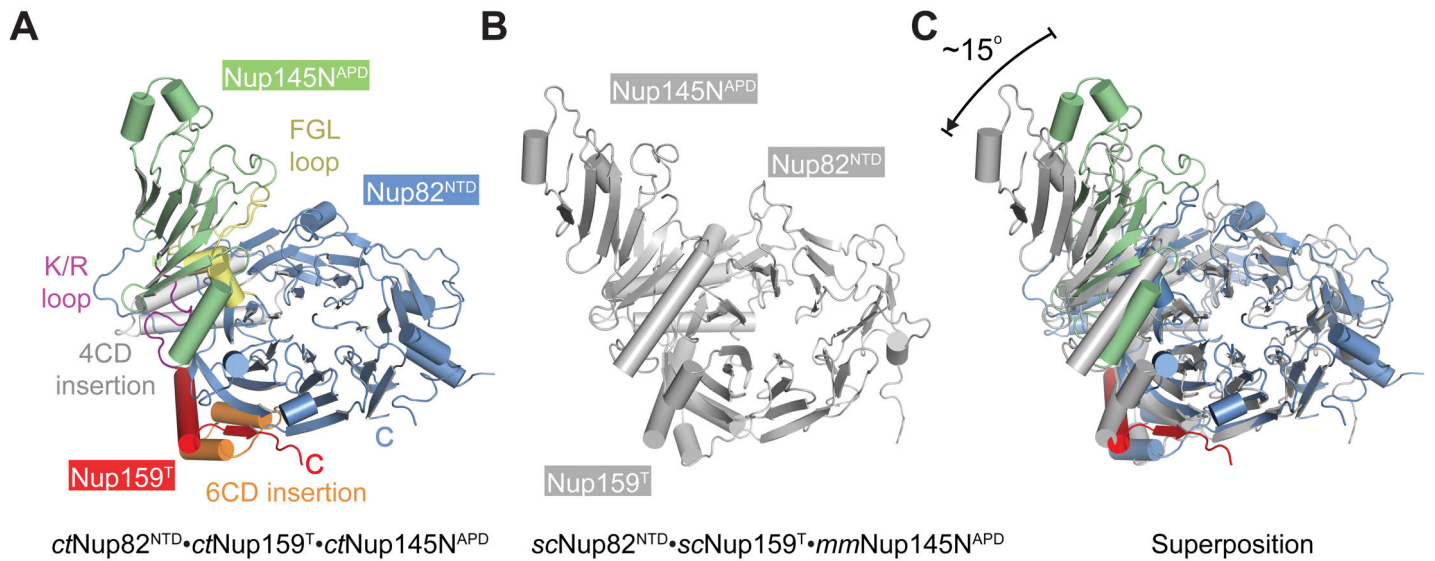




**Fig. S2.**

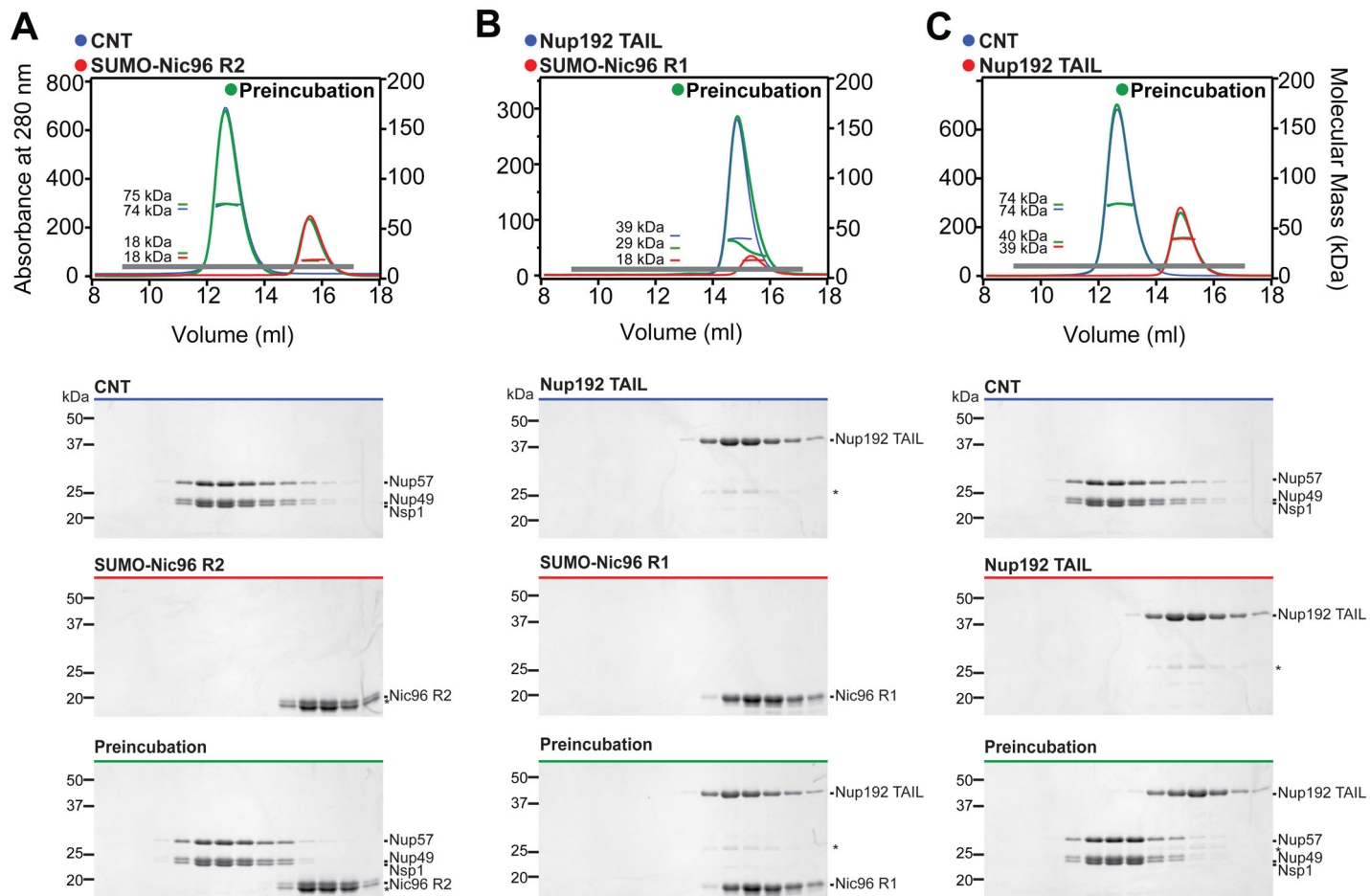
**Reconstitution and dissection of the IRC (continued).** (A to F) SEC-MALS and SDS-PAGE analysis corresponding to [Figure 1](#). SEC-MALS profiles of nucleoporins or nup complexes are shown individually (red and blue) and after their preincubation (green). Measured molecular masses are indicated for the peak fractions. Gray bars indicate fractions that were resolved on SDS-PAGE gels and visualized by Coomassie staining.





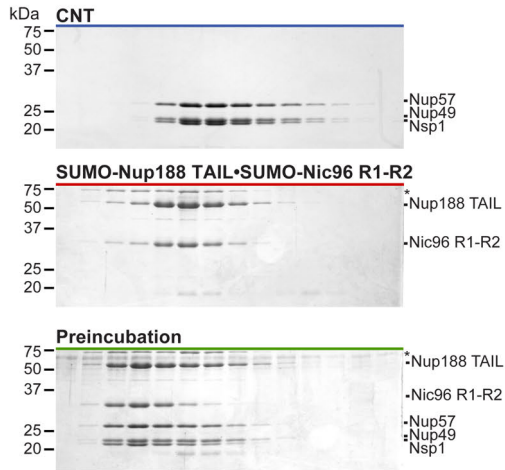
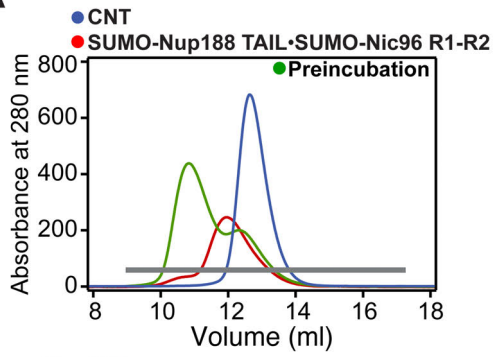
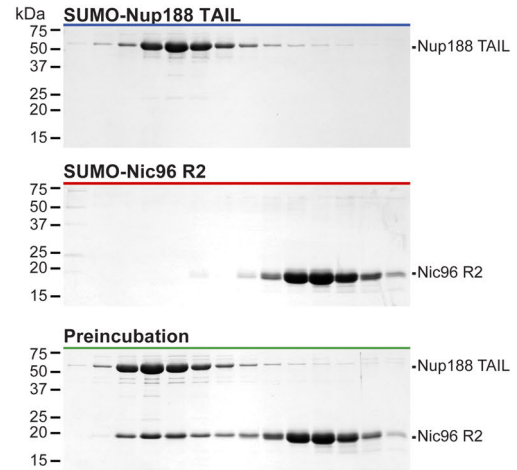
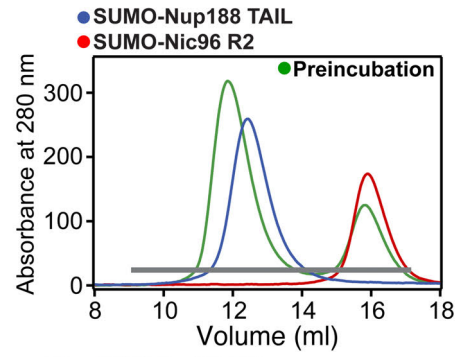
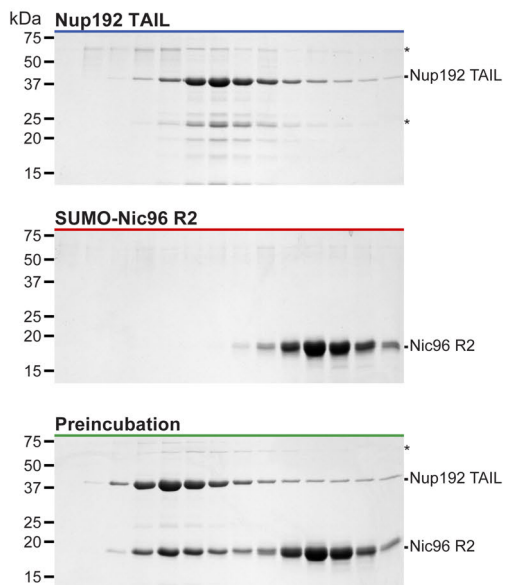
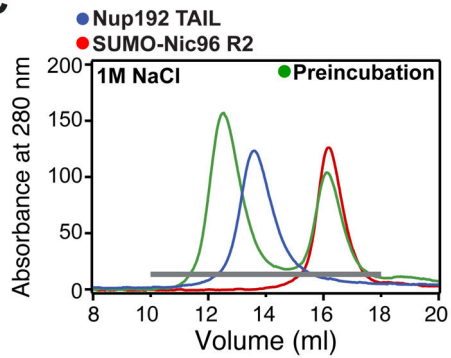
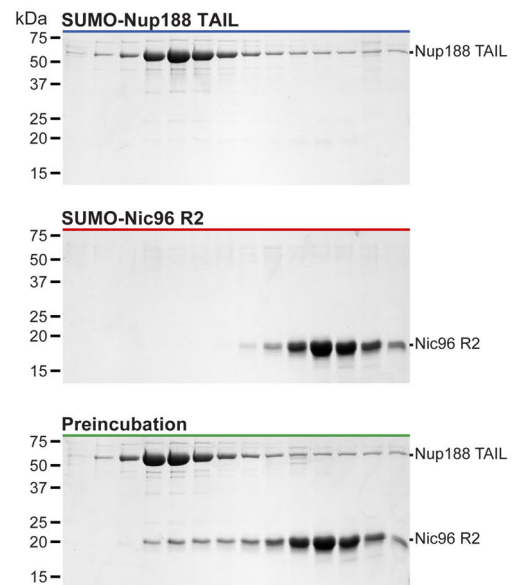
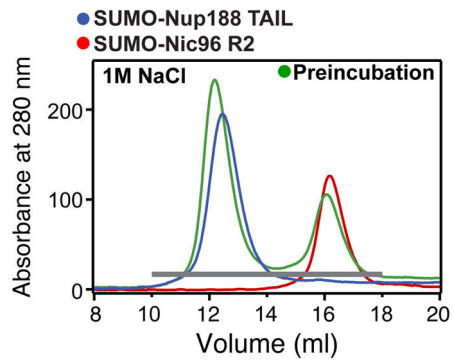
**Fig. S3.**

**Structure of the minimal CFC in complex with Nup145N<sup>APD</sup>.** (A) Structure of the Nup82<sup>NTD</sup>•Nup159<sup>T</sup>•Nup145N<sup>APD</sup> hetero-trimer shown in cartoon representation. Nup145N<sup>APD</sup> (green), Nup159<sup>T</sup> (red), and Nup82<sup>NTD</sup> (blue), the Nup82<sup>NTD</sup> helical 4CD (grey) and 6CD (orange) insertions and FGL loop (yellow), and the conserved Nup145N<sup>APD</sup> K loop (purple) are illustrated. (B) Structure of the previously determined scNup82<sup>NTD</sup>•scNup159<sup>T</sup>•mmNup145N<sup>APD</sup> hetero-trimer (PDB ID: 3TKN) shown in cartoon representation (29). (C) Superposition of the two hetero-trimers in cartoon representation, revealing the evolutionary conservation of their architectures, and differing mainly by an angular displacement of Nup145<sup>APD</sup>. (D) The *Inset* marks the location of the two close-up views, depicting the molecular details of the Nup145N<sup>APD</sup>-Nup82<sup>NTD</sup> interaction (top) and the Nup159<sup>T</sup>-Nup82<sup>NTD</sup> interaction (bottom). The D pocket that binds the K loop is indicated. (E) Sequence alignments of the conserved *S. cerevisiae* and *C. thermophilum* Nup159<sup>T</sup>, the Nup82<sup>NTD</sup> FGL loop, and the Nup145N K/R loop. Sequence similarity is shaded from white (less than 60 % similarity), to yellow (60 % similarity), to red (100 % identity) using the BLOSUM62 matrix. Numbering below alignment is according to the *C. thermophilum* proteins. Secondary structure is shown above the alignment.



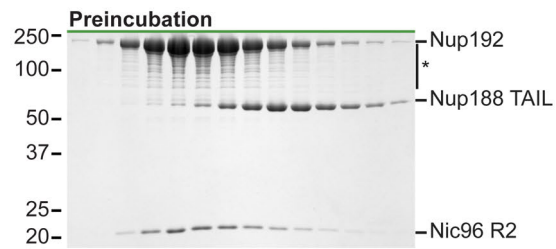
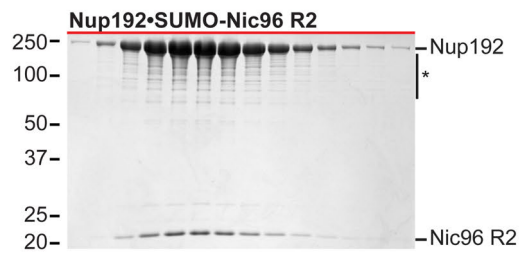
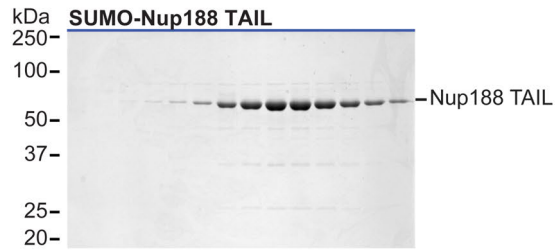
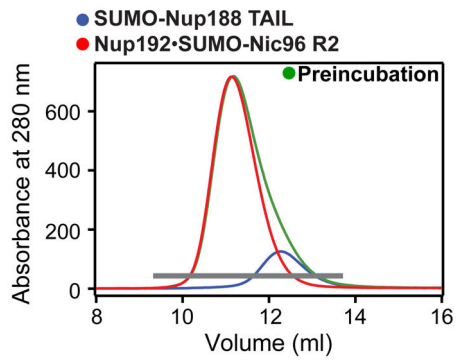
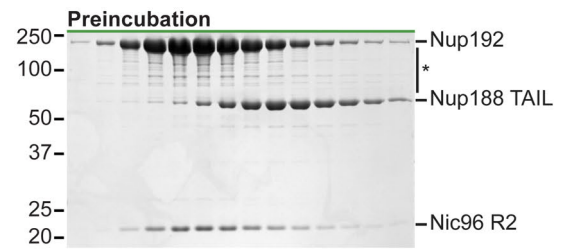
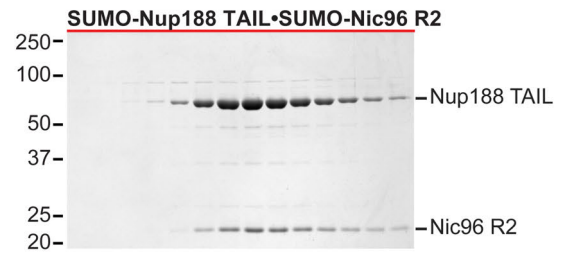
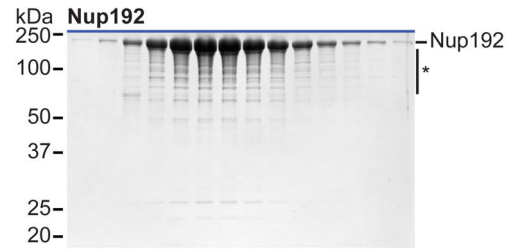
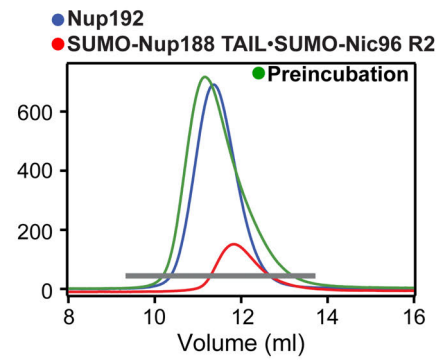
**Fig. S4.**

**The CNT does not interact with Nic96<sup>R2</sup> or Nup192<sup>TAIL</sup>, nor does Nic96<sup>R1</sup> bind Nup192<sup>TAIL</sup>.** (A to C) SEC-MALS profiles of nucleoporins or nup complexes are shown individually (red and blue) and after their preincubation (green). Measured molecular masses are indicated for the peak fractions. Gray bars indicate fractions that were resolved on SDS-PAGE gels and visualized by Coomassie staining. Asterisks indicate degradation products or contamination.

**A****B****C****D**

**Fig. S5.**

**Nup188<sup>TAIL</sup> forms an alternative complex with the CNT.** (A) Reconstitution of SUMO-Nup188<sup>TAIL</sup>•SUMO-Nic96<sup>R1-R2</sup>•CNT. (B) Reconstitution of SUMO-Nup188<sup>TAIL</sup>•SUMO-Nic96<sup>R2</sup>. (C, D) SEC analysis of Nup192<sup>TAIL</sup>•SUMO-Nic96<sup>R2</sup> and SUMO-Nup188<sup>TAIL</sup>•SUMO-Nic96<sup>R2</sup> carried out in a 1 M NaCl buffer. SEC profiles of nucleoporins or nup complexes are shown individually (red and blue) and after their preincubation (green). Gray bars indicate fractions that were resolved on SDS-PAGE gels and visualized by Coomassie staining. Asterisks indicate degradation products or contamination.

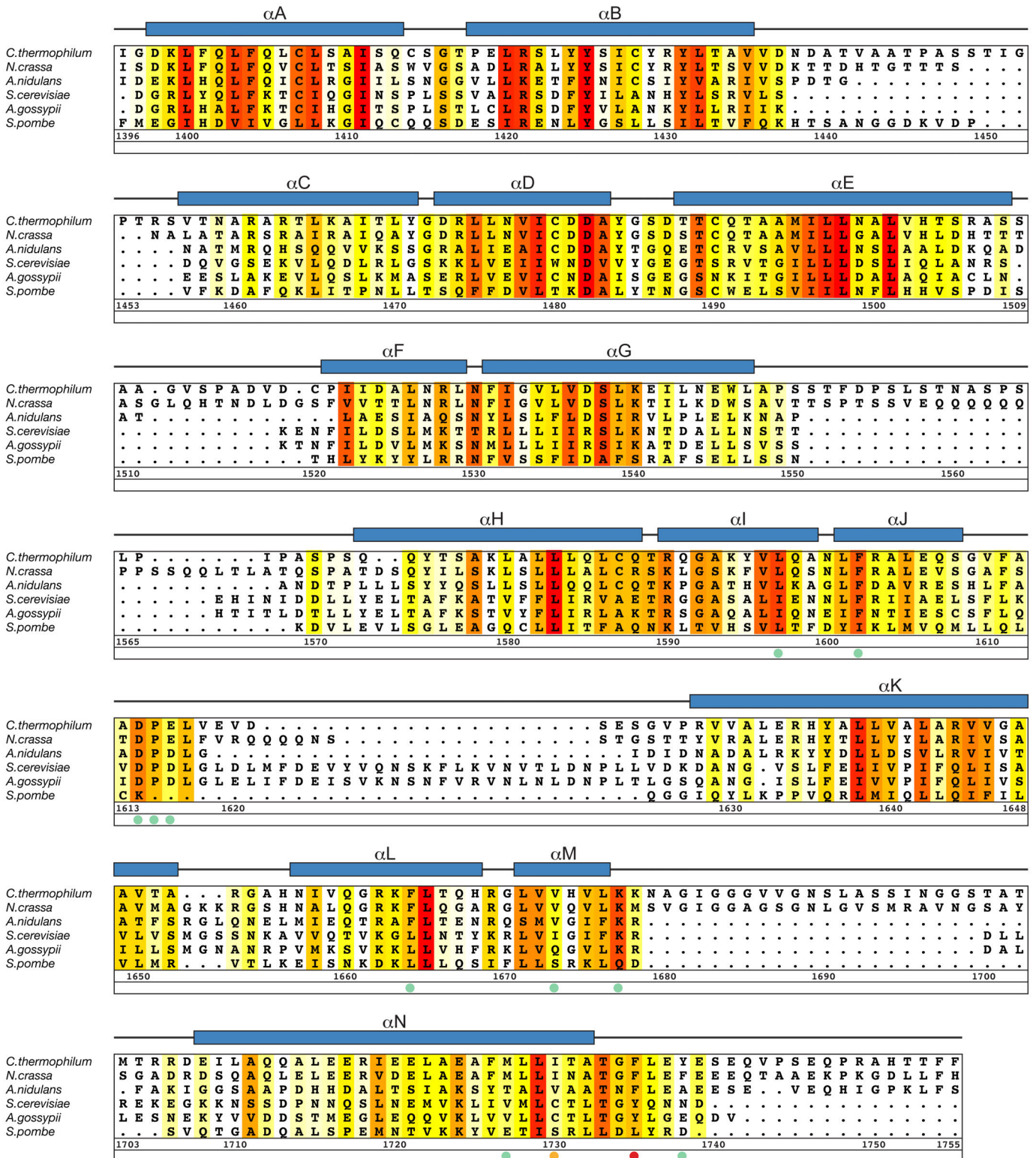
**A****B**



**Fig. S6.**

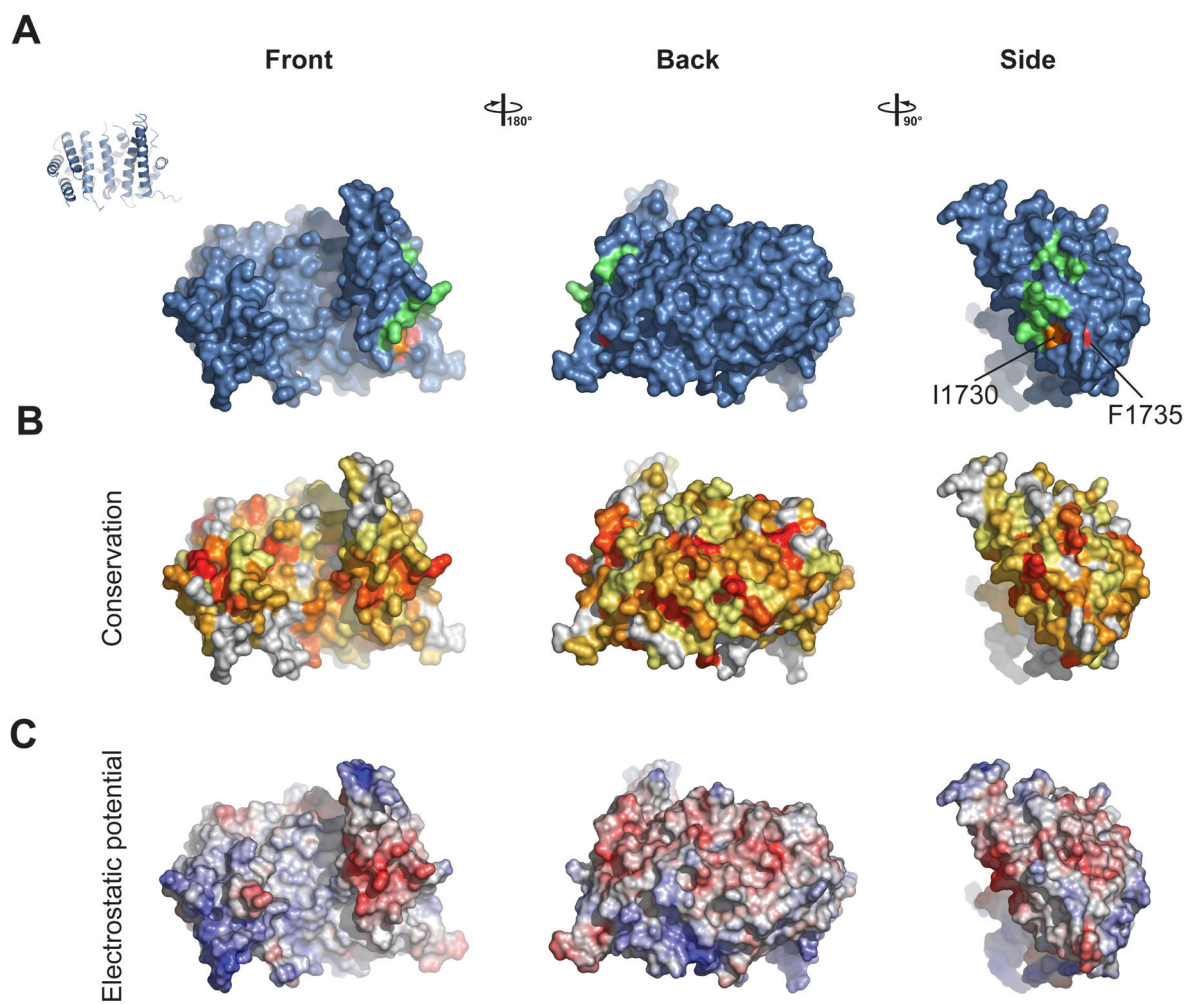
**Binding of Nup192 and Nup188 to Nic96 is mutually exclusive.** (A) SEC interaction analysis of SUMO-Nup188<sup>TAIL</sup> challenging a preformed Nup192•SUMO-Nic96<sup>R2</sup> complex. (B) SEC interaction analysis of Nup192 challenging a preformed SUMO-Nup188<sup>TAIL</sup>•SUMO-Nic96<sup>R2</sup> complex. SEC profiles of nucleoporins or nup complexes are shown individually (red and blue) and after their preincubation (green). Proteins were mixed at equimolar ratios. Gray bars indicate fractions that were resolved on SDS-PAGE gels and visualized by Coomassie staining. Asterisks indicate degradation products or contamination.

# Nup192 TAIL



**Fig. S7.**

**Multispecies sequence alignment of Nup192<sup>TAIL</sup>.** Sequences from six diverse fungal species were aligned and colored by sequence similarity according to the BLOSUM62 matrix from white (less than 40 % similarity), to yellow (40 % similarity), to red (100 % identity). Numbering below alignment is relative to *C. thermophilum*. Secondary structure observed in the Nup192<sup>TAIL</sup> structure is shown above the alignment;  $\alpha$ -helices (blue bars) and black lines (unstructured regions). Residues tested for their ability to interact with Nic96 in a mutational analysis ([Figures 2](#) and [S12](#)) are indicated with dots below the alignment and colored according to the measured effect; no effect (green), mild effect (orange), abolished binding (red).

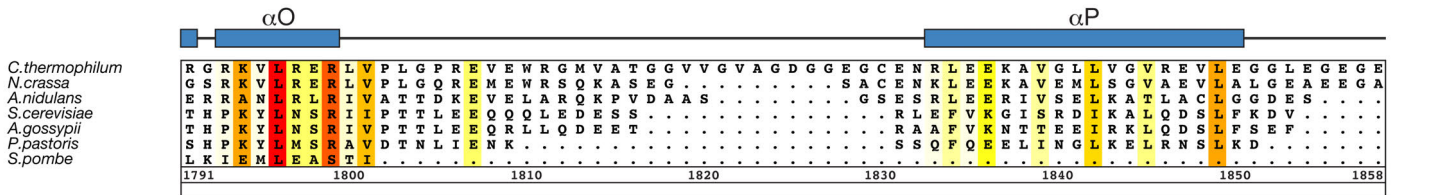
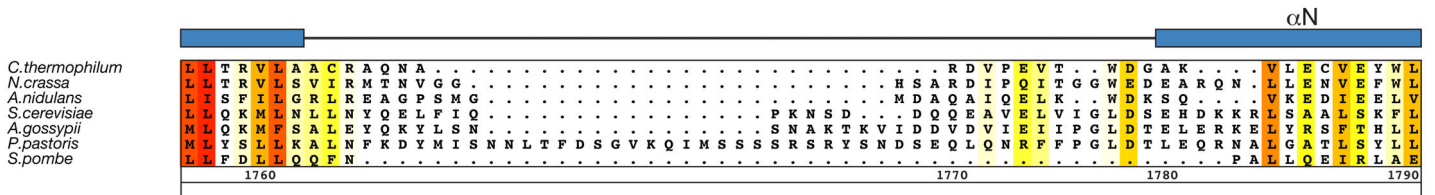
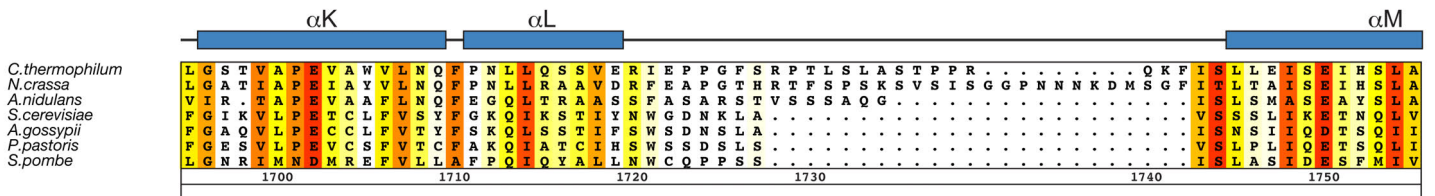
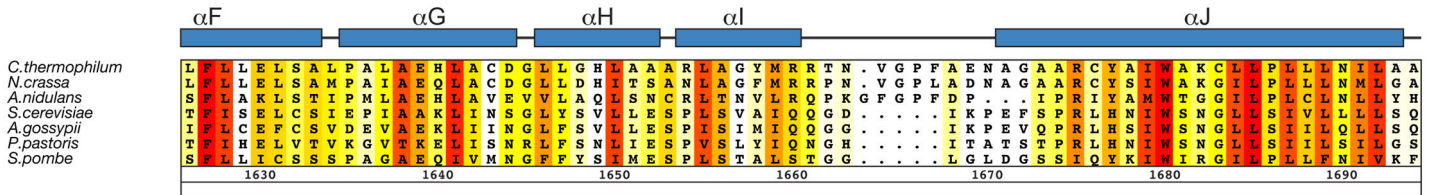
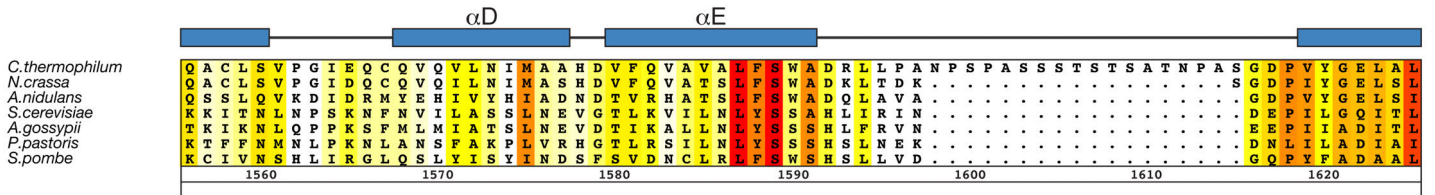
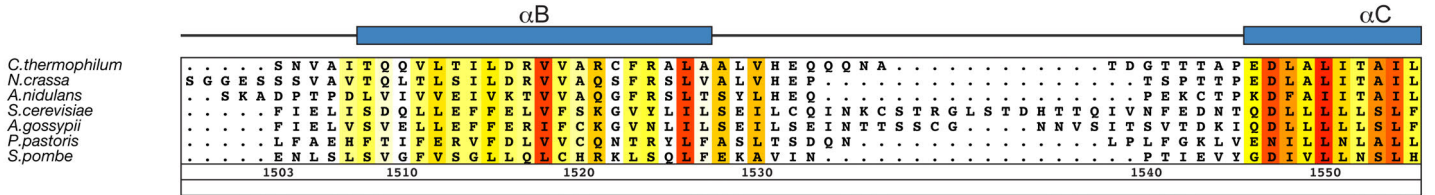
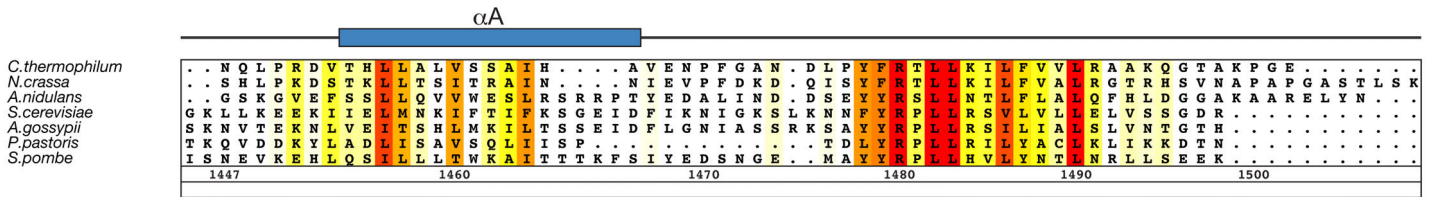


**Fig. S8.**

**Surface properties of Nup192<sup>TAIL</sup>.** Surface representation of Nup192<sup>TAIL</sup> is shown in three different orientations, front, back and side. The degree of rotation around the vertical axis is indicated between the respective views. **(A)** Mutated Nup192<sup>TAIL</sup> residues are colored according to their effect on SUMO-Nic96<sup>R2</sup> binding; no effect (green), mild effect (orange), abolished binding (red). **(B)** Surface representation colored according to sequence identity based on the alignment in [Figure S7](#). **(C)** Surface representation colored according to electrostatic potential from -10 k<sub>B</sub>T/e (red) to +10 k<sub>B</sub>T/e (blue).



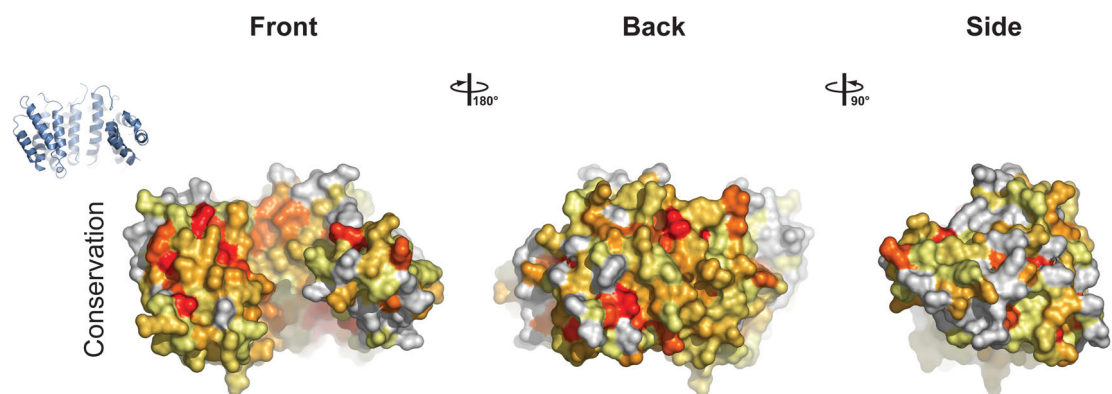
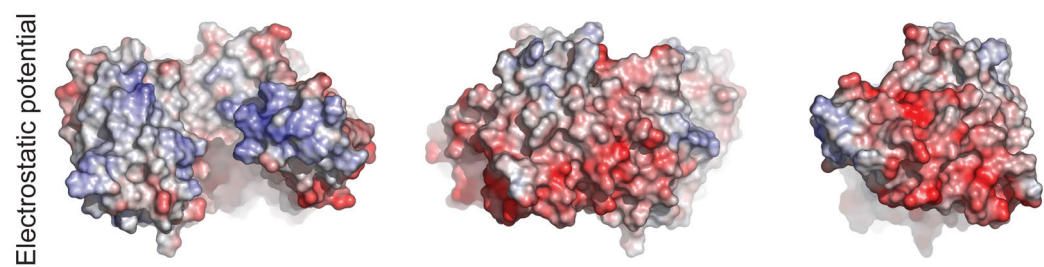
# Nup188 TAIL



**Fig. S9.**

**Multispecies sequence alignment of Nup188<sup>TAIL</sup>.** Sequences from seven diverse fungal species were aligned and colored by sequence similarity according to the BLOSUM62 matrix from white (less than 40 % similarity), to yellow (40 % similarity), to red (100 % identity). Numbering below alignment is relative to *C. thermophilum*. Secondary structure observed in the Nup188<sup>TAIL</sup> structure is shown above the alignment;  $\alpha$ -helices (blue bars) and black lines (unstructured regions).



**A****B**

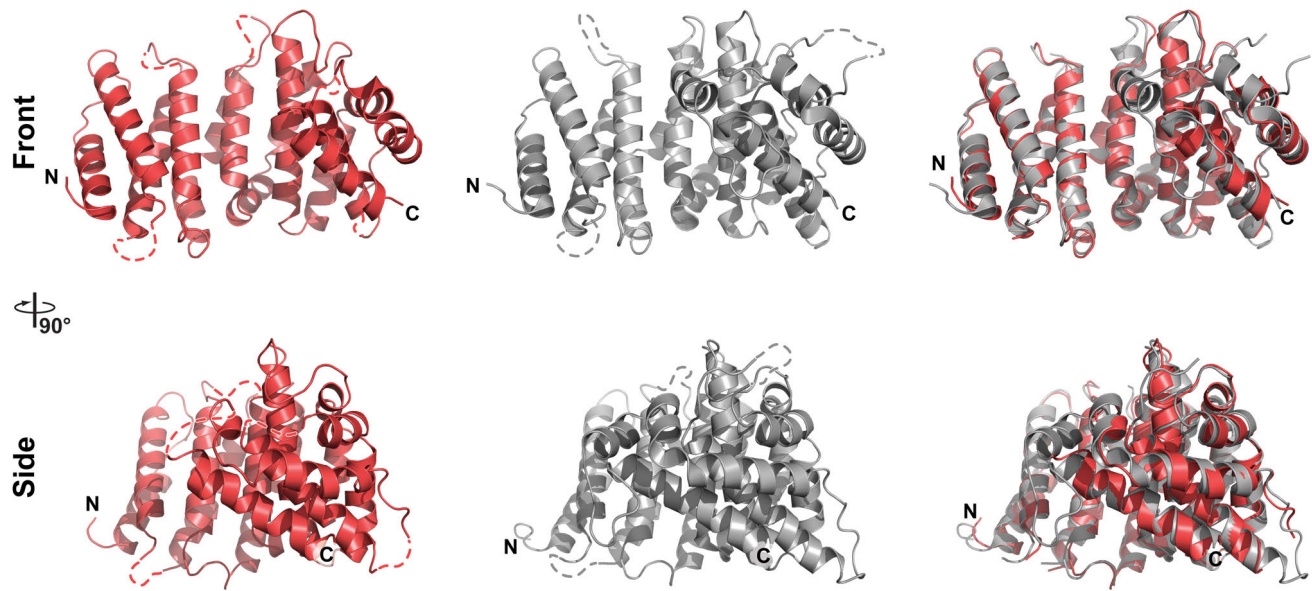
**Fig. S10.**

**Surface properties of Nup188<sup>TAIL</sup>.** Surface representation of Nup188<sup>TAIL</sup> is shown in three different orientations, front, back and side. Rotations around the vertical axis are indicated between the respective views. **(A)** Surface representation colored according to sequence identity based on alignment in [Figure S9](#). **(B)** Surface representation colored according to electrostatic potential from  $-10\text{ k}_\text{B}T/e$  (red) to  $+10\text{ k}_\text{B}T/e$  (blue).

*C.thermophilum* Nup188 TAIL

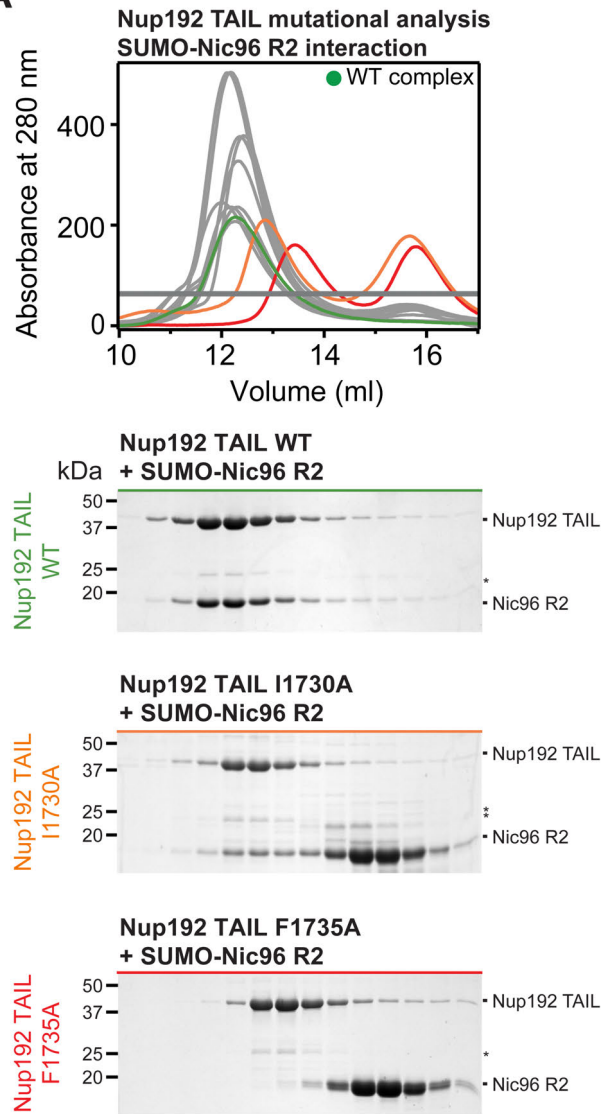
*M.thermophila* Nup188 TAIL

Superposition



**Fig. S11.**

**Comparison of *ct*Nup188<sup>TAIL</sup> and *mt*Nup188<sup>TAIL</sup> structures.** Cartoon representations of (A) *C. thermophilum* Nup188<sup>TAIL</sup> (red), (B) *M. thermophila* Nup188<sup>TAIL</sup> (grey) (PDB ID: 4KF8) (31), and (C) a superposition of the two structures is shown in two different orientations.

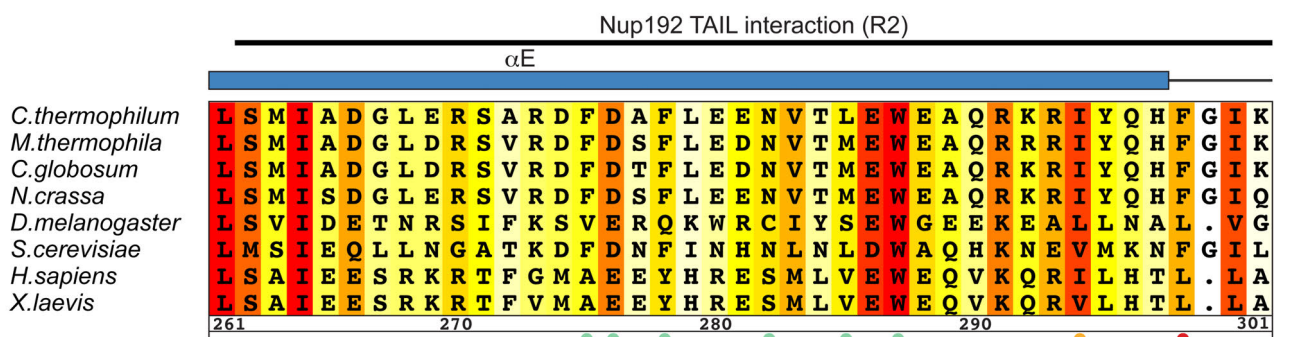
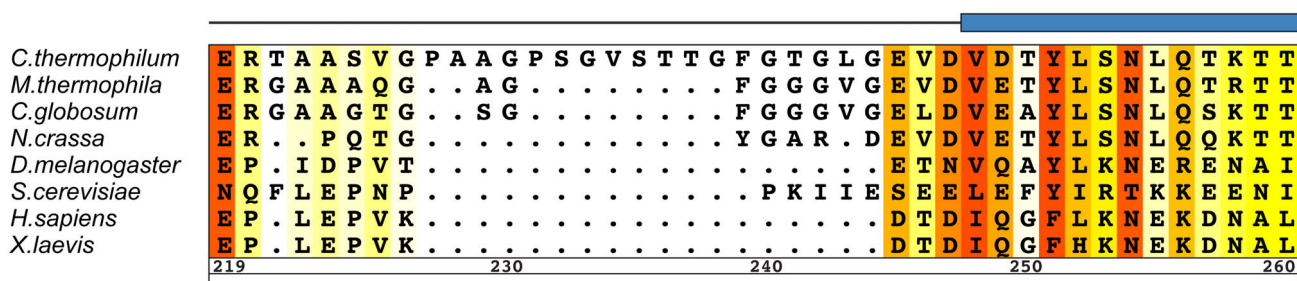
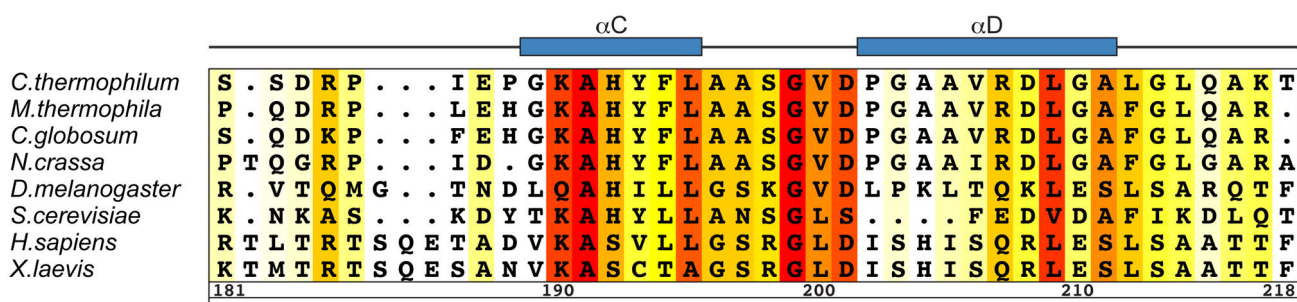
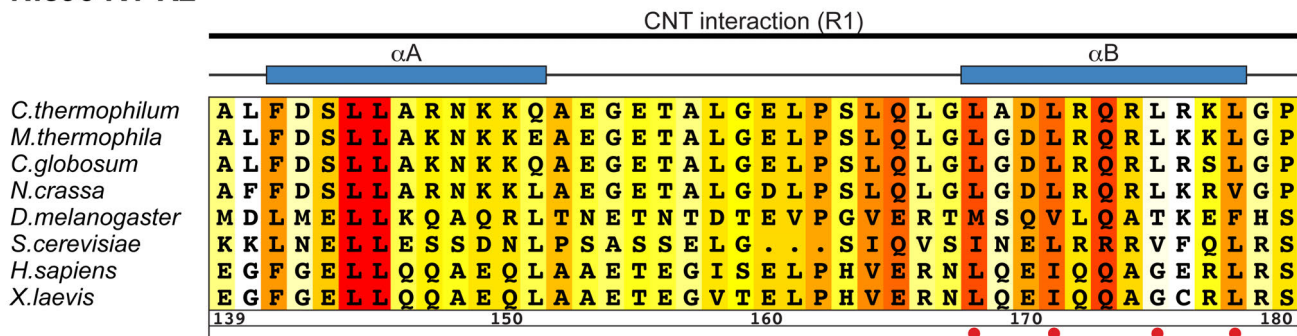
**A****B**

Nup192 TAIL mutations	
Mutation	effect
● L1597A	-
F1602A	insoluble
● F1602W	-
F1611A	insoluble
● DPE1614,15,16AAA	-
● DPE1614,15,16APA	-
F1664A	insoluble
● F1664W	-
● V1673A	-
L1676A	insoluble
● K1677A	-
● M1727A	-
● I1730A	+
● F1735A	+++
● Y1738A	-

**Fig. S12.**

**Identification of Nup192<sup>TAIL</sup> mutations that disrupt SUMO-Nic96<sup>R2</sup> binding. (A)** SEC interaction analysis between Nup192<sup>TAIL</sup> mutants and SUMO-Nic96<sup>R2</sup>. SEC profiles are colored according to the effect on SUMO-Nic96<sup>R2</sup> binding; no effect (gray), mild effect (orange), or abolished binding (red). As reference, the wild type complex is shown (green). A gray bar indicates fractions that were resolved on SDS-PAGE gels and visualized by Coomassie staining. **(B)** Summary of tested Nup192<sup>TAIL</sup> mutants and their effect on SUMO-Nic96<sup>R2</sup> binding; (-) no effect, (+) decreased binding, (+++) abolished binding. The colors of the dots correspond to the color of the respective SEC profile in (A).

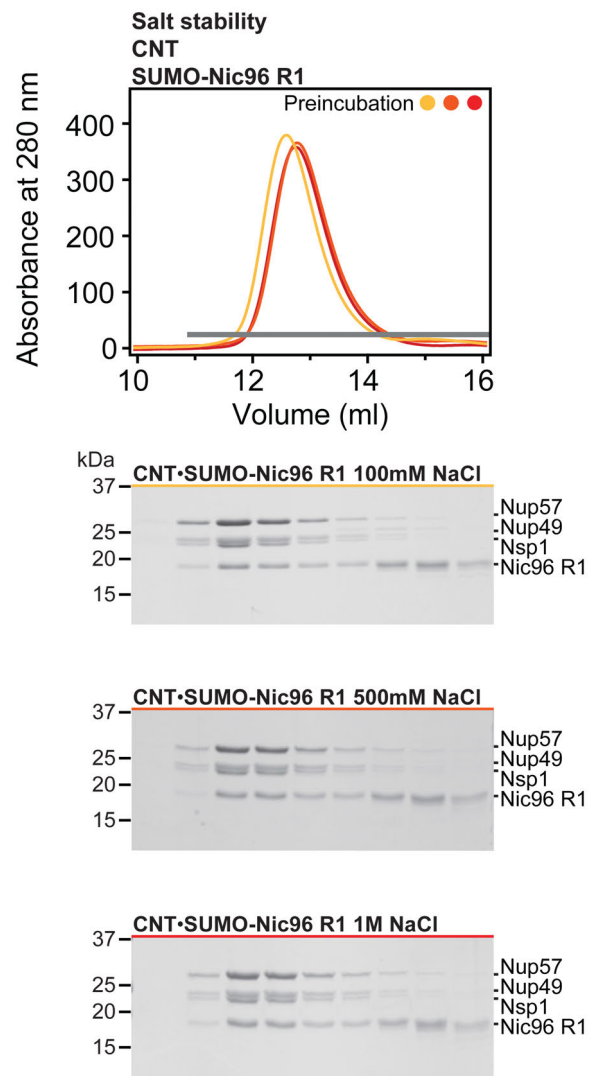
## Nic96 R1-R2





**Fig. S13.**

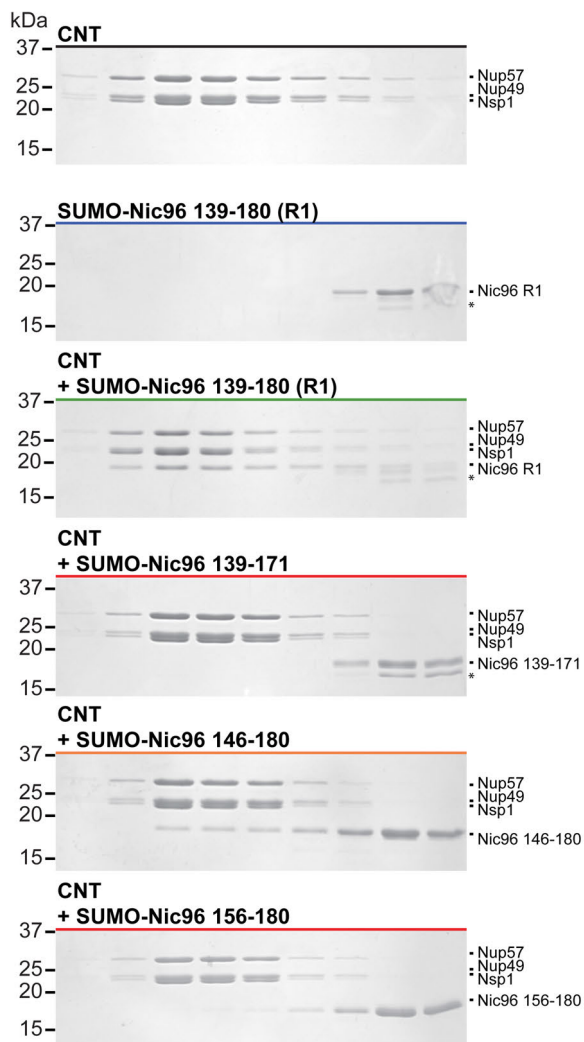
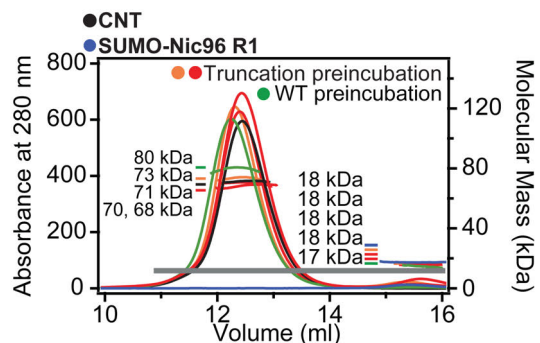
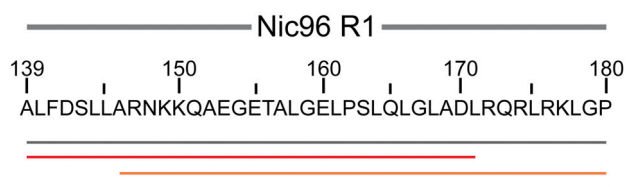
**Multispecies sequence alignment of Nic96<sup>R1-R2</sup>.** Sequences from eight diverse species were aligned and colored by sequence similarity according to the BLOSUM62 matrix from white (less than 40 % similarity), to yellow (40 % similarity), to red (100 % identity). Numbering below alignment is relative to *C. thermophilum*. The secondary structure is shown above the alignment;  $\alpha$ -helices are represented by blue bars. Black bars above the alignment indicate CNT-binding (R1) and Nup192-binding regions (R2). Dots below the alignment indicate the positions of mutations tested for CNT or Nup192<sup>TAIL</sup> binding ([Figures 3, S15, and S16](#)); no effect (green), mild effect (orange), abolished binding (red).



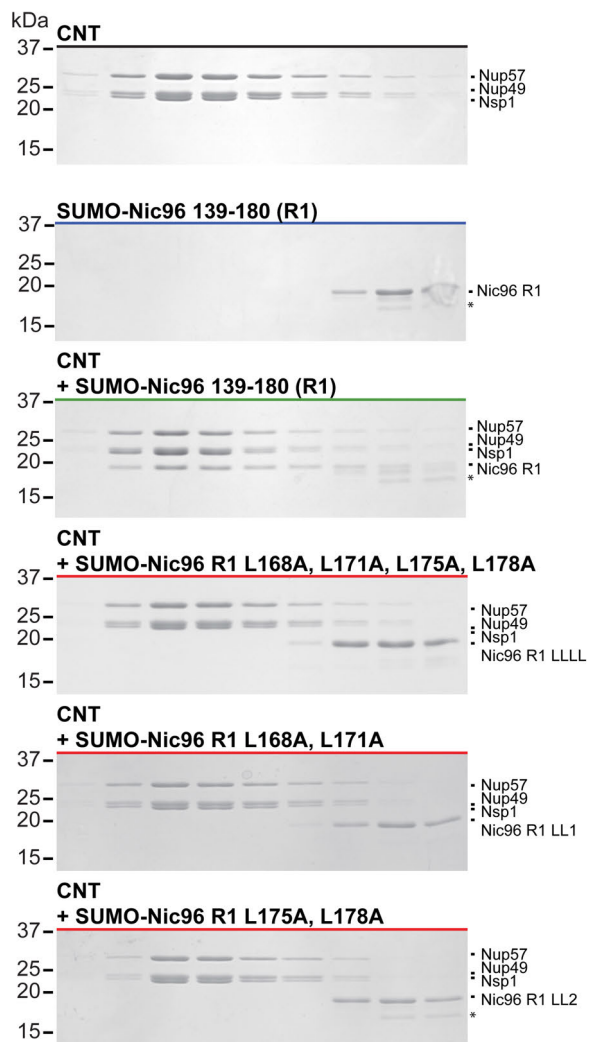
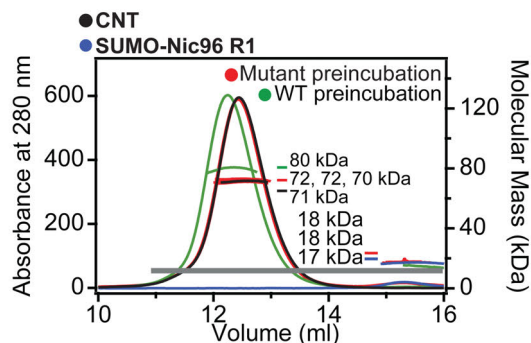
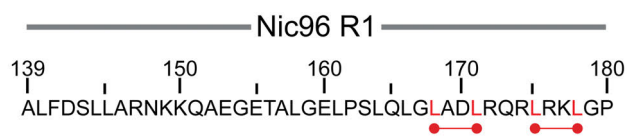
**Fig. S14.**

**Salt stability of the CNT•SUMO-Nic96<sup>R1</sup> complex.** SEC analysis of purified CNT•SUMO-Nic96<sup>R1</sup> carried out in a buffer containing 0.1 M (yellow), 0.5 M (light orange) or 1.0 M NaCl (red). A gray bar indicates fractions that were resolved on a SDS-PAGE gel and visualized by Coomassie staining.

**A**



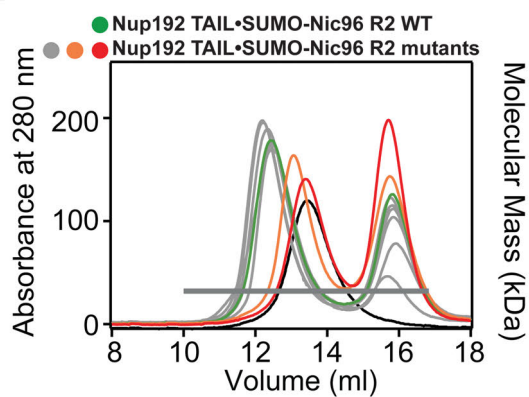
**B**



**Fig. S15.**

**Mapping and mutational analysis of the minimal Nic96<sup>R1</sup> region required for CNT binding. (A, B)** Analyzed Nic96<sup>R1</sup> truncations and Nic96<sup>R1</sup> leucine-alanine mutants are indicated below the primary Nic96<sup>R1</sup> sequence. SEC-MALS profiles of SUMO-Nic96<sup>R1</sup> fragments or mutants preincubated with CNT are shown and colored according to their effect on complex formation; mild effect (orange) or abolished binding (red). As reference, SEC profiles of CNT (black) and SUMO-Nic96<sup>R1</sup> (blue) and their preincubation (green) are shown. Measured molecular masses are indicated for the peak fractions. Gray bars indicate fractions that were resolved on SDS-PAGE gels and visualized by Coomassie staining. LL1, LL2, and LLLL refer to the three combinations of SUMO-Nic96<sup>R1</sup> leucine-alanine mutants tested. Asterisks indicate degradation products or contamination.

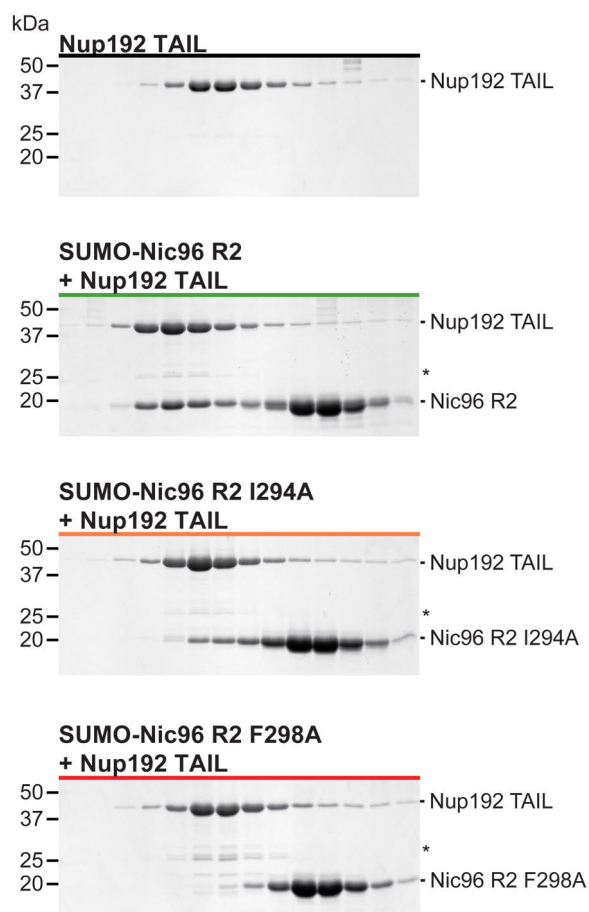
**A**



**B**

**Nic96 R2 mutations**

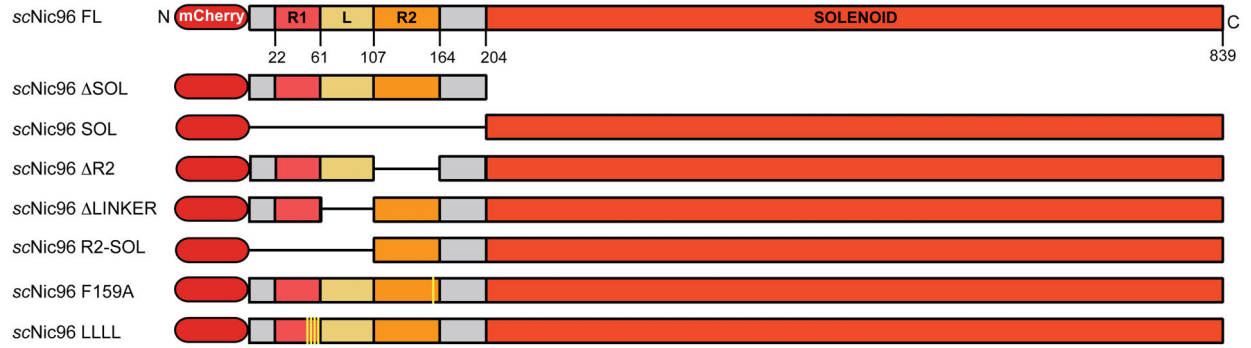
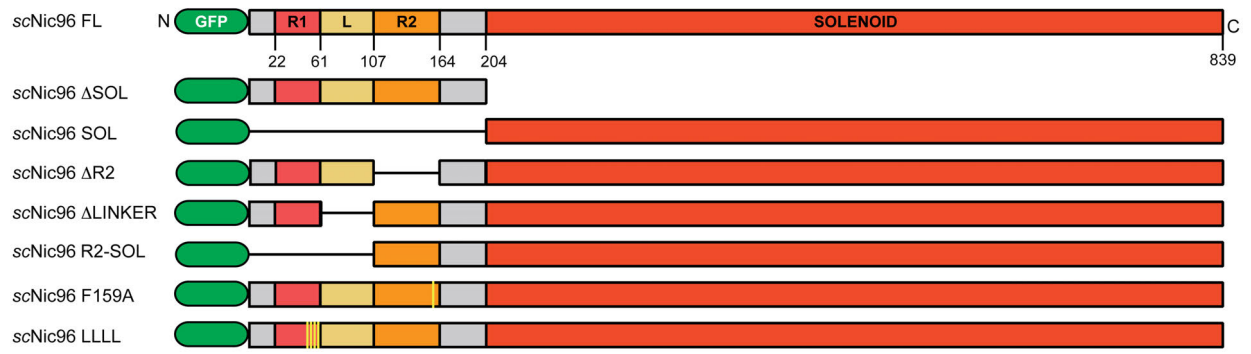
Mutation	effect
● F275A	-
● D276A	-
● F278A	-
● N282A	-
● L285A	-
● W287A	-
● I294A	+
● F298A	+++





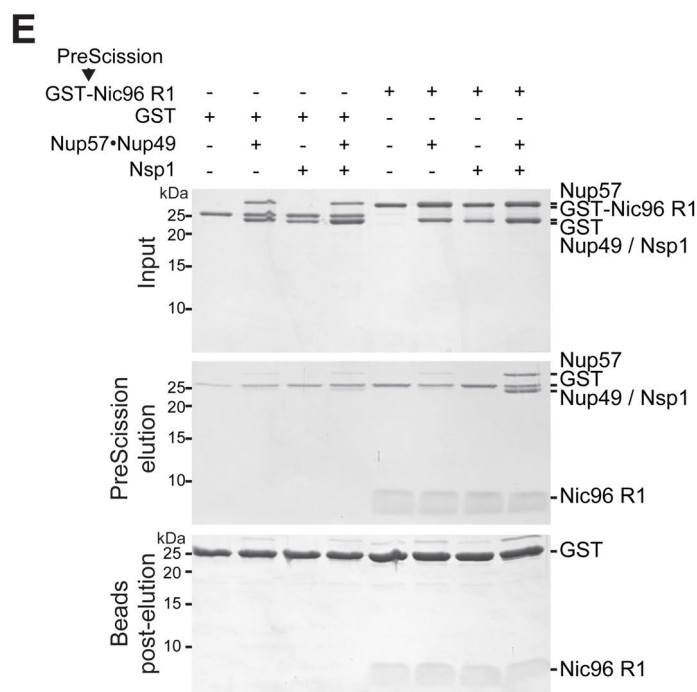
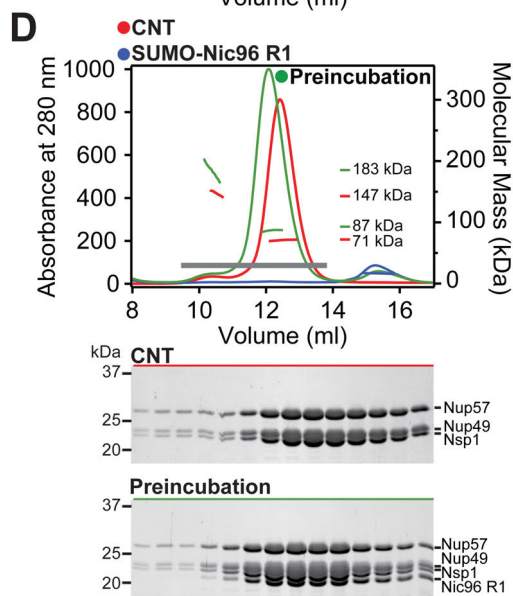
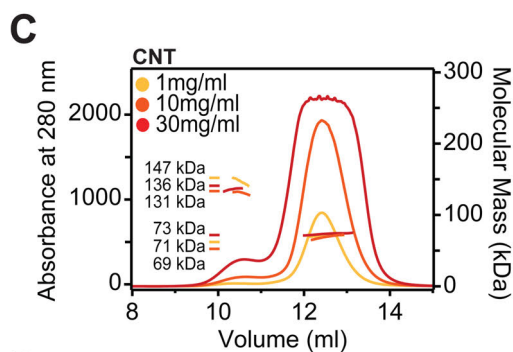
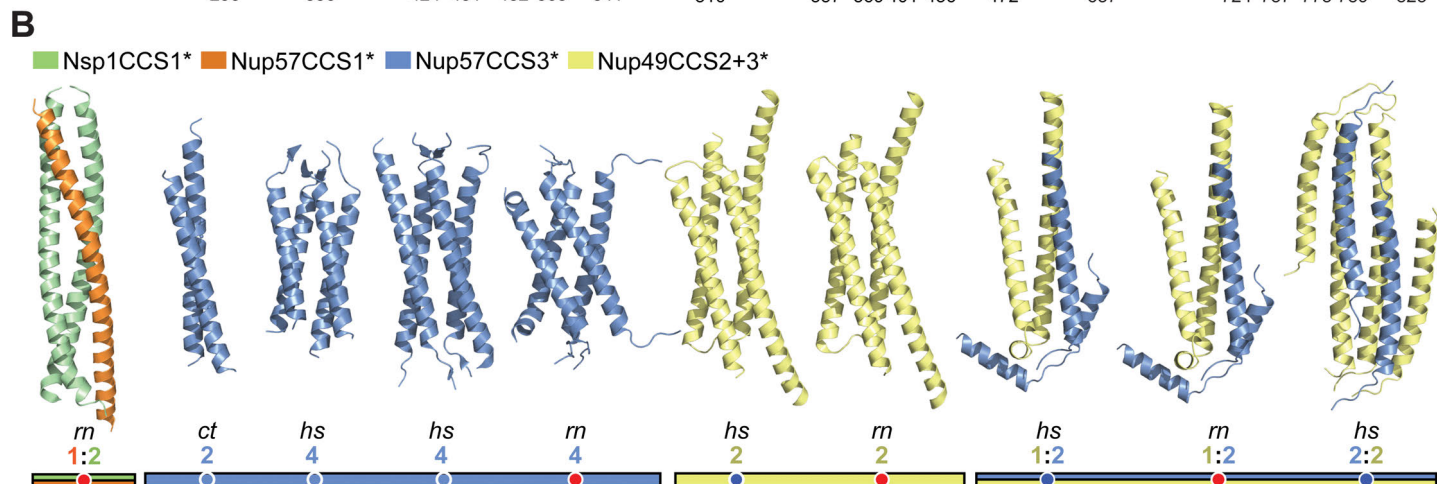
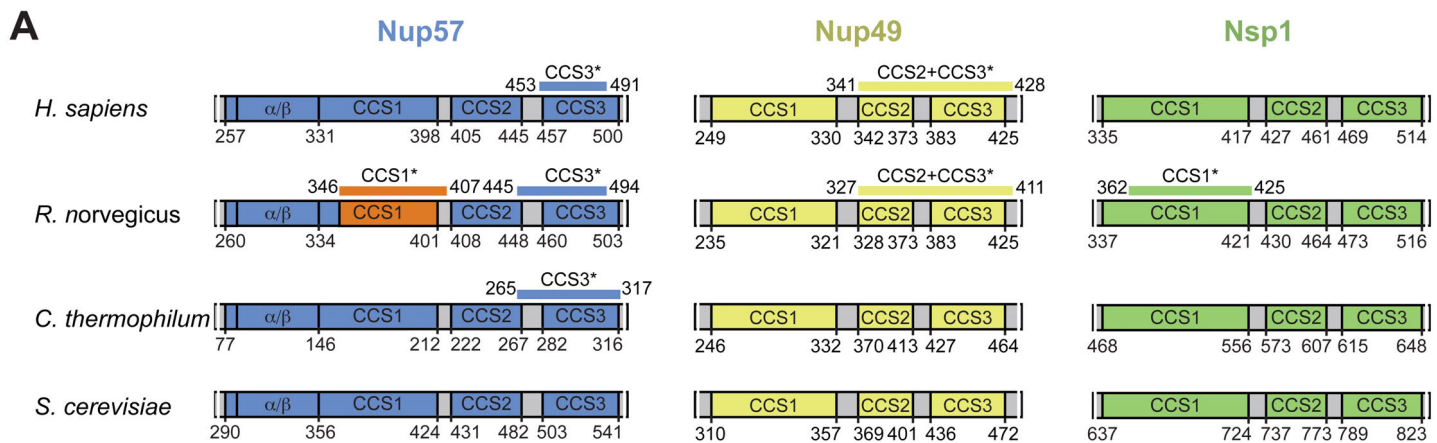
**Fig. S16.**

**Identification of Nic96<sup>R2</sup> mutations that disrupt Nup192<sup>TAIL</sup> binding.** (A) SEC interaction analysis between SUMO-Nic96<sup>R2</sup> mutants and Nup192<sup>TAIL</sup>. SEC profiles are colored according to the effect on Nup192<sup>TAIL</sup> binding; no effect (gray), mild effect (orange), or abolished binding (red). For reference, SEC profiles of wild type Nup192<sup>TAIL</sup>•SUMO-Nic96<sup>R2</sup> (green) and Nup192<sup>TAIL</sup> (black) are shown. A gray bar indicates fractions that were resolved on SDS-PAGE gels and visualized by Coomassie staining. (B) Summary of tested SUMO-Nic96<sup>R2</sup> mutants and their effect on Nup192<sup>TAIL</sup> binding; (-) no effect, (+) decreased binding, (+++) abolished binding. The colors of the dots correspond to the color of the respective SEC profile in (A).



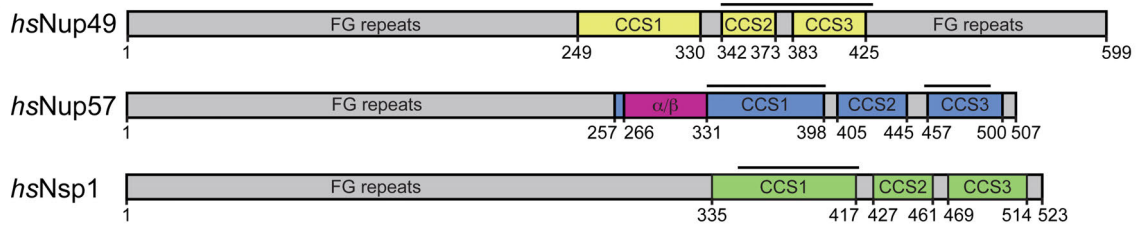
**Fig. S17.**

***S. cerevisiae* GFP-Nic96 and mCherry-Nic96 constructs used for *in vivo* analyses.** The domain architecture of *S. cerevisiae* Nic96 is shown and colored according to [Figure 1](#). Black lines indicate the regions that were removed in various Nic96 deletion mutants. The positions of the F159A and LLLL mutants that abolish Nup192 and CNT binding, respectively, are highlighted in yellow.



**Fig. S18.**

**Characterization of channel nucleoporin fragments and the intact CNT.** (A) Domain structures of the three CNT-forming channel nups from *H. sapiens*, *R. norvegicus*, and *C. thermophilum*. Bars indicate the boundaries of the crystallized Nup57 (blue, orange), Nup49 (yellow), and Nsp1 (green) fragments. (B) Crystal structures of seven channel nup fragments in isolation or in complex colored according to (A). The species and fragment stoichiometry are indicated for each structure. For clarity, only the homo-dimers of *H. sapiens* and *R. norvegicus* Nup49<sup>CCS2+3\*</sup> are shown. The structures of Nup57<sup>CCS3\*</sup>, hsNup57<sup>CCS3\*</sup> (two different states), hsNup49<sup>CCS2+3\*</sup>, and hsNup49<sup>CCS2+3\*</sup>•hsNup57<sup>CCS3\*</sup> (with 1:2 and 2:2 stoichiometry) reported here are indicated with blue dots. The previously determined structure of rnNup57<sup>CCS1\*</sup>•rnNsp1<sup>CCS1\*</sup> (1:2 stoichiometry), rnNup57<sup>CCS3\*</sup>, rnNup49<sup>CCS2+3\*</sup>, and rnNup49<sup>CCS2+3\*</sup>•rnNup57<sup>CCS3\*</sup> (with 1:2 stoichiometry) are marked with red dots (22, 25, 26). (C) SEC-MALS profiles of the CNT at three different concentrations (yellow to red). Asterisks indicate that the crystallized channel nup fragments do not exactly correspond to the predicted coiled-coil regions. (D) SEC-MALS interaction analysis of CNT (red), SUMO-Nic96<sup>R1</sup> (blue), and after their preincubation (green). Measured molecular masses are indicated for the peak fractions. Gray bars indicate fractions that were resolved on SDS-PAGE gels and visualized by Coomassie staining. (E) GST pull-down interaction analysis of Nsp1, Nup49•Nup57 or the premixed CNT with GST-Nic96<sup>R1</sup> or GST as bait. The input, PreScission protease elution, and post-elution beads were analyzed by SDS-PAGE and Coomassie staining. Nic96<sup>R1</sup> specifically pulls-down the intact CNT and fails to interact with Nsp1 or Nup49•Nup57.

**A****B**

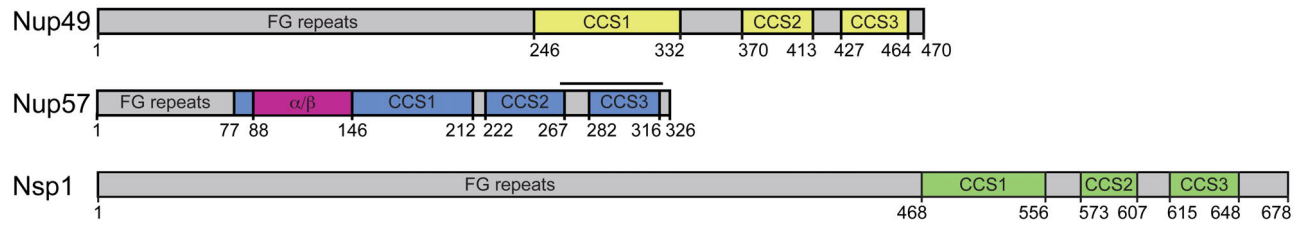
		GST		His <sub>6</sub>		His <sub>6</sub> -SUMO	
		Expression	Solubility	Expression	Solubility	Expression	Solubility
<i>hsNup49</i>	250-299	++	++	-	N/A	+++	+++
	250-331	+++	++	-	N/A	+++	+++
	250-375	+++	+	++	-	+++	+++
	250-430	+++	+	+++	-	+++	++
	302-331	+++	++	-	N/A	+++	+++
	302-375	+++	++	++	-	++	++
	302-430	+++	++	+++	+	+++	+++
	341-375	+++	++	-	N/A	++	++
	341-430	+++	++	++	+	+++	+++
	383-430	+++	-	-	N/A	-	N/A
<i>hsNup57</i>	● 341-428	+++	++	++	-	+++	++
	331-364	++	++	-	N/A	++	++
	331-399	++	++	-	N/A	++	+
	331-450	+++	-	++	-	++	-
	331-493	+++	-	+++	-	+++	-
	343-364	+++	++	+	-	++	++
	● 343-399	+	++	-	N/A	+++	-
	343-450	+++	-	++	-	+++	-
	343-493	+++	-	+++	-	+++	-
	365-399	+	++	-	N/A	+	++
<i>hsNsp1</i>	365-450	++	-	++	-	++	-
	365-493	+++	-	+++	-	+++	-
	404-450	++	++	-	N/A	+	++
	404-493	+++	++	++	+	+++	++
	456-493	+++	+++	-	N/A	++	++
	● 453-491	+++	++	-	N/A	+++	+++
	322-367	++	++	+	N/A	++	++
	322-420	+++	++	+	++	+++	++
	322-461	++	++	++	-	+	++
	322-522	+++	-	++	-	+++	-
<i>hsNic96</i>	370-420	+	++	-	N/A	++	++
	370-461	+	++	-	N/A	+	++
	370-522	+	-	++	-	+++	-
	427-461	+	++	-	N/A	+	++
	427-522	+++	-	++	-	+++	++
	469-522	+++	-	+	-	+++	++
	● 359-422	++	+++	-	N/A	++	++
	1-80	++	++	+	++	-	N/A
	1-151	+++	+	++	-	+++	+
	96-151	+++	-	+	-	++	++

● crystallized

**Fig. S19.**

**Expression and solubility analysis of *H. sapiens* channel nups.** (A) Domain architecture of the three human channel nups Nup49 (yellow), Nup57 (blue/purple), and Nsp1 (green) are shown. (B) The expression and solubility levels of individual CNT-fragments expressed as GST-fusions, hexahistidine (HIS<sub>6</sub>) or HIS<sub>6</sub>-SUMO tagged proteins are indicated; not expressed, insoluble (-) to highly expressed, highly soluble (+++). Crystallized fragments are highlighted with a black bar and a black dot.



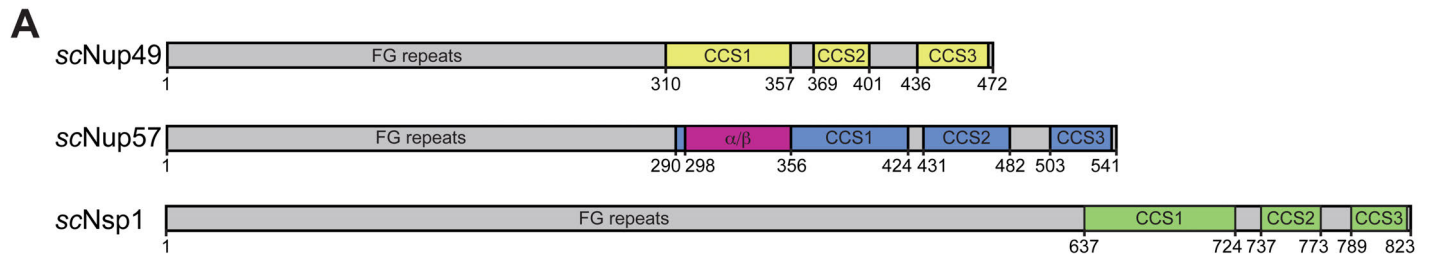
**A****B**

	GST		His <sub>6</sub>		His <sub>6</sub> -SUMO	
	Expression	Solubility	Expression	Solubility	Expression	Solubility
Nup49	246-281	++	-	N/A	+	+++
	246-333	++	-	N/A	+	+++
	246-404	++	+	-	+++	+++
	246-470	++	+++	-	+++	+++
	285-333	++	+	-	+++	+++
	285-404	++	+	-	++	++
	285-470	++	++	-	+++	+
	370-404	+++	-	N/A	+++	++
	370-470	+++	+++	-	+++	+
	427-470	+++	-	N/A	+	++
Nup57	74-143	+	+	-	+	-
	74-220	++	++	-	+	-
	74-269	+++	+++	-	++	-
	74-317	+++	+++	-	++	-
	74-326	+++	+++	-	+++	+
	143-220	+++	-	N/A	++	-
	143-269	+++	++	-	+	-
	143-317	+++	+++	-	+++	+
	143-326	+++	+++	-	+	++
	158-220	+++	-	N/A	++	-
	158-269	+++	+	-	+++	+
	158-317	+++	++	++	+++	++
	158-326	+++	++	++	+++	++
	221-269	+++	-	N/A	++	++
	221-317	+++	+	+++	++	++
	221-326	+++	+	++	+++	+++
Nsp1	● 265-317	+++	-	N/A	+++	+++
	265-326	+++	-	N/A	+++	+++
	58-216	++	-	N/A	++	++
	272-310	+++	-	N/A	+++	+++
	467-501	++	-	N/A	+	++
	467-555	+++	-	N/A	++	++
	467-605	+++	+	+++	+++	++
	467-653	+++	++	+	+++	++
	467-678	+++	++	++	+++	++
	504-555	++	+	-	+++	++
	504-605	+++	++	-	++	++
	504-653	+++	+	+++	+++	++
	504-678	+++	+++	-	+++	++
	569-605	+++	-	N/A	++	++
	569-653	+++	+	-	+++	++
	569-678	+++	+	-	+++	++
	615-653	+++	-	N/A	+	++
Nic96	615-678	+++	-	N/A	++	++
	495-552	+-++	-	N/A	+++	+++
	110-153	+++	++	-	+++	+++
	110-180	++	+++	-	++	+++
	110-211	+	++	N/A	+++	+++
	110-301	+++	+++	-	++	++
	139-180	++	-	N/A	++	++
	139-211	+	-	N/A	++	+++
	139-301	+++	++	-	++	++
	187-301	+++	+	-	++	++
	245-301	+++	+	-	+++	++

●crystallized

**Fig. S20.**

**Expression and solubility analysis of *C. thermophilum* channel nups.** (A) Domain architecture of the three *C. thermophilum* channel nups are shown and colored according to [Figure S19](#). (B) The expression and solubility levels of individual CNT-fragments expressed as GST-fusions, hexahistidine (HIS<sub>6</sub>) or HIS<sub>6</sub>-SUMO tagged proteins are indicated; not expressed, insoluble (-) to highly expressed, highly soluble (+++). Crystallized fragments are highlighted with a black bar and a black dot.

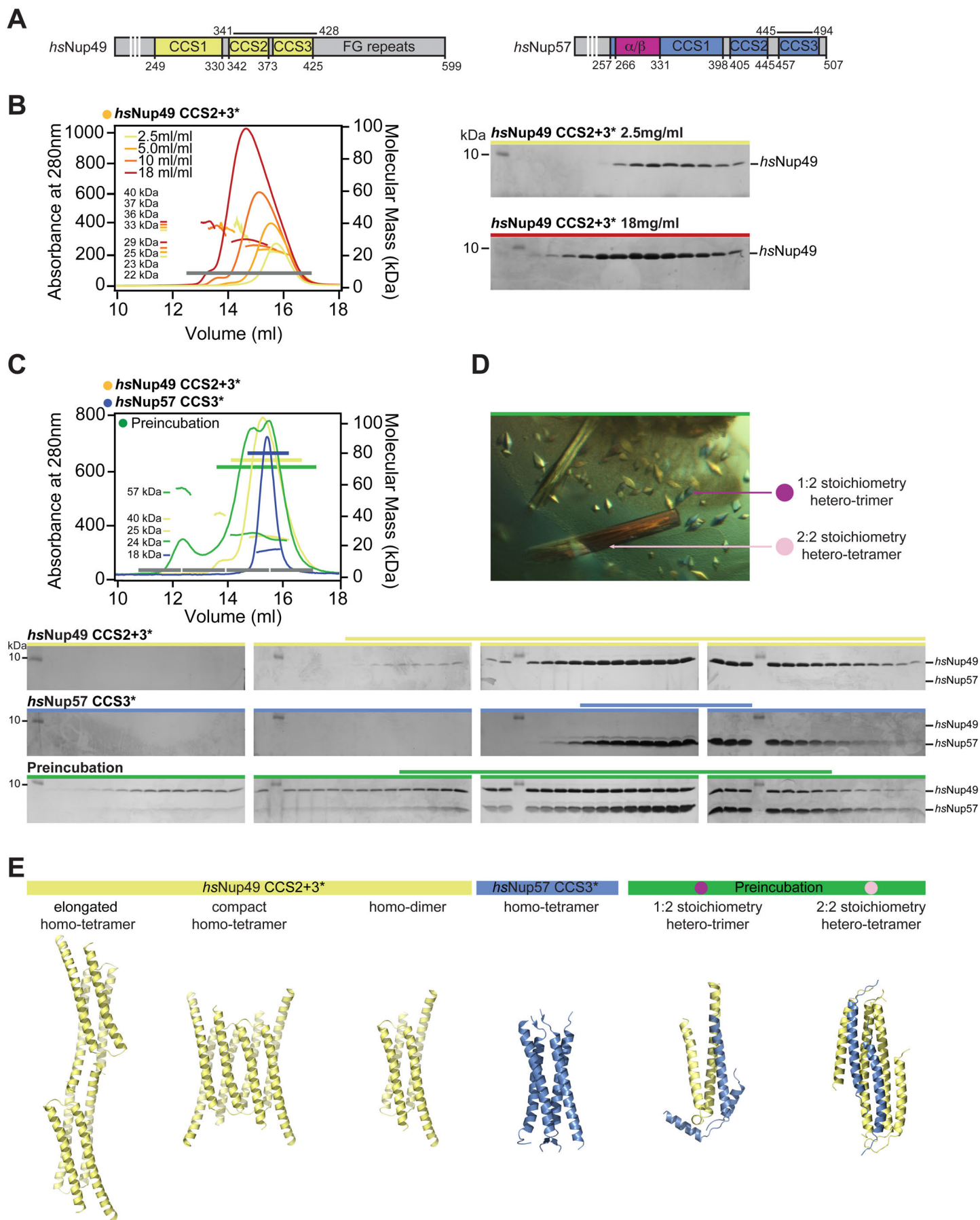


**B**

		GST		His <sub>6</sub>		His <sub>6</sub> -SUMO	
		Expression	Solubility	Expression	Solubility	Expression	Solubility
scNup49	310-356	++	+	++	-	+	++
	310-405	+++	++	+++	-	++	+
	310-472	+++	+	+++	-	+++	-
	366-405	+++	++	+++	-	++	++
	366-471	+++	+	+++	-	++	++
	425-472	+++	++	-	N/A	-	N/A
scNup57	333-389	+++	++	+	-	+++	+++
	333-425	+++	-	+++	-	++	-
	333-487	+++	+	+++	-	+++	+
	333-541	+++	-	+++	-	++	++
	356-389	+++	+++	+	-	+++	+++
	356-425	++	+	++	-	++	++
	356-487	+++	+	++	-	+++	+
	356-541	+++	+	+++	-	++	++
	391-425	+++	+++	+	-	+++	+++
	391-487	+	-	++	-	+++	-
	391-541	+++	++	++	-	++	+++
	433-487	++	++	+	-	+	+++
	433-541	+++	++	+	-	++	-
	500-541	+++	+++	+	+	+++	+++
scNsp1	368-425	+++	++	-	N/A	++	+
	486-524	++	++	-	N/A	+++	+++
	623-682	+	++	-	N/A	+++	++
	623-727	++	++	+	+++	+++	++
	623-773	+	++	+	-	+	++
	623-823	+++	-	+	-	+++	-
	649-682	+	++	-	N/A	++	++
	649-727	++	++	+	-	+++	+++
	649-773	+	++	+	-	+	++
	649-823	+++	+	++	-	+++	++
	694-727	+++	++	-	N/A	++	+++
	694-773	+	++	-	N/A	+	+++
	694-823	+++	++	+	++	+++	++
	738-773	+	++	-	N/A	+	++
scNic96	738-823	++	+	++	-	+++	++
	787-823	+++	++	-	N/A	+++	++
	665-722	+	++	-	N/A	++	++
	1-60	+++	-	+	-	++	++
	1-160	+++	-	+++	-	+++	+
	16-60	++	++	++	-	++	++
	16-160	+++	-	+++	-	+++	+
	107-160	++	+	++	-	++	++

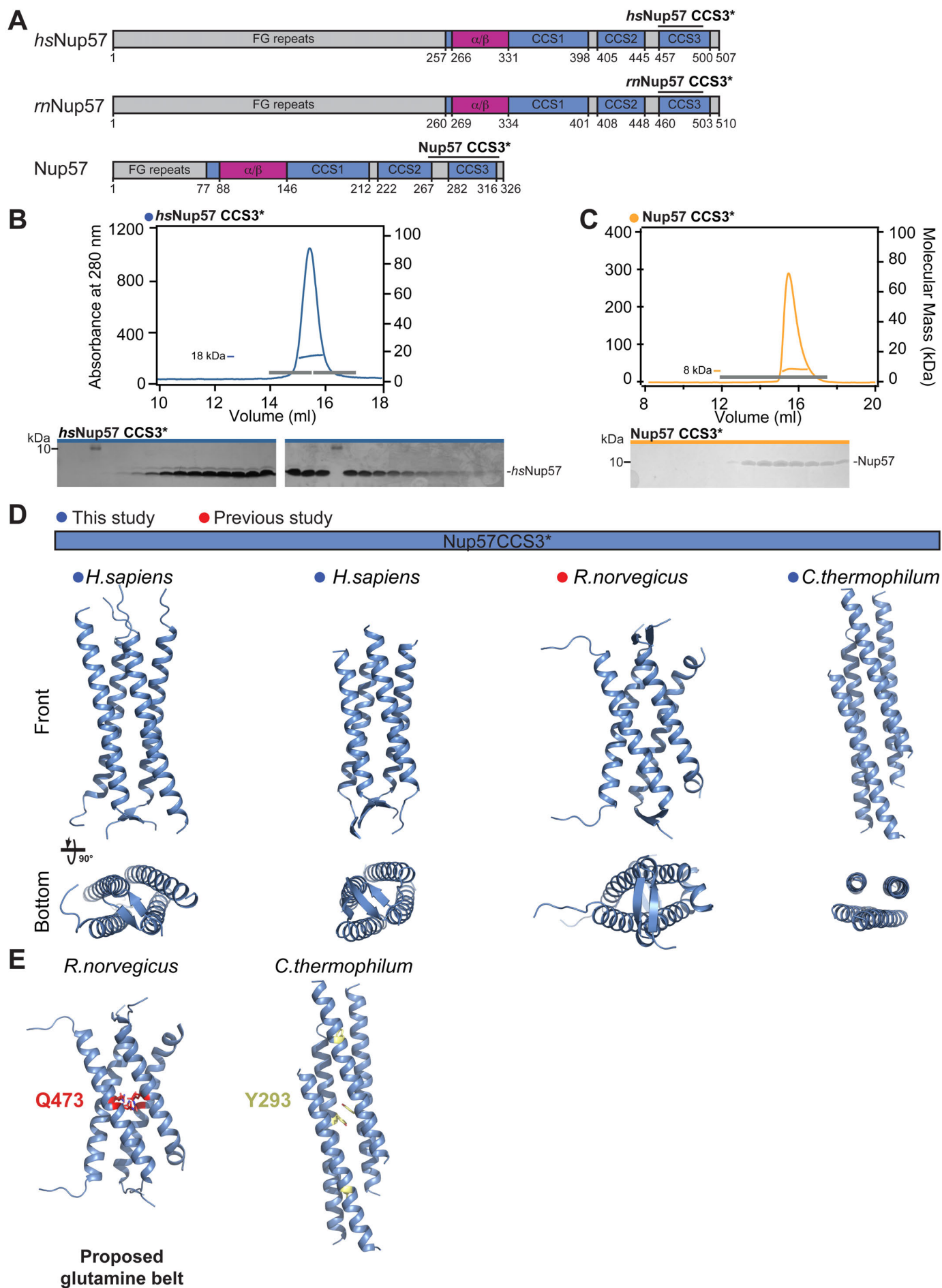
**Fig. S21.**

**Expression and solubility analysis of *S. cerevisiae* channel nups.** (A) Domain architecture of the three *S. cerevisiae* channel nups are shown and colored according to [Figure S19](#). (B) The expression and solubility levels of individual CNT-fragments expressed as GST-fusions, hexahistidine (HIS<sub>6</sub>) or HIS<sub>6</sub>-SUMO tagged proteins are indicated; not expressed, insoluble (-) to highly expressed, highly soluble (+++). Crystallized fragments are highlighted with a black bar and a black dot.



**Fig. S22.**

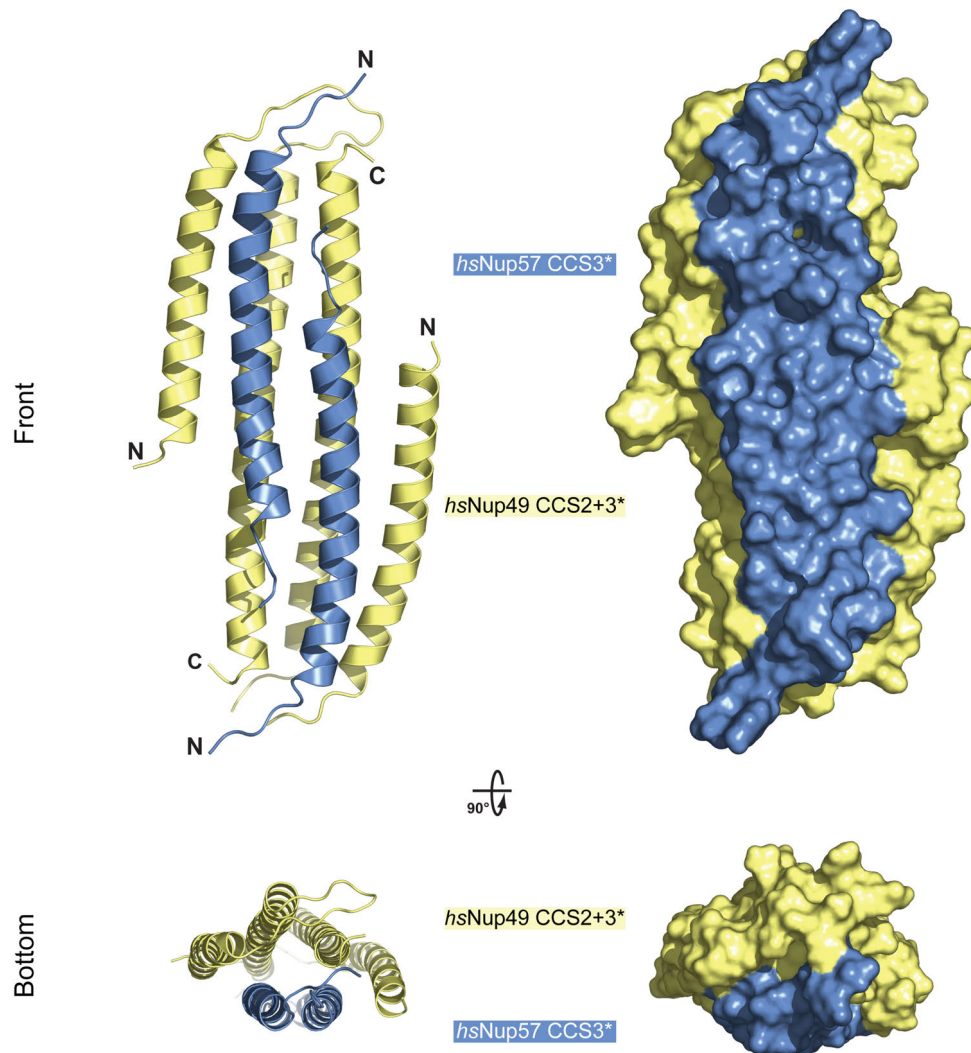
**Biochemical and structural analysis of different *hsNup49*<sup>CCS2+3\*</sup> and *hsNup57*<sup>CCS3\*</sup> complexes reveal different oligomerization states.** (A) Domain architecture of *hsNup49* and *hsNup57* is shown and colored according to [Figure S19](#). (B) SEC-MALS profiles of *hsNup49*<sup>CCS2+3\*</sup> are shown at increasing protein concentrations (yellow to red). (C) SEC-MALS profiles of *hsNup57*<sup>CCS3\*</sup> and *hsNup49*<sup>CCS2+3\*</sup> are shown individually (blue and yellow) and after their preincubation (green). Measured molecular masses are indicated for the peak fractions. Gray bars indicate fractions that were resolved on SDS-PAGE gels and visualized by Coomassie staining. Colored bars indicate the fractions that were pooled for crystallization. (D) Crystallization drop showing two different crystal forms of the *hsNup57*<sup>CCS3\*</sup>•*hsNup49*<sup>CCS2+3\*</sup> complex with 1:2 (purple) and 2:2 (magenta) stoichiometry. (E) The structure of the three different homo-oligomers of *hsNup49*<sup>CCS2+3\*</sup> (elongated homo-tetramer, compact homo-tetramer, and homo-dimer), the homo-tetramer of *hsNup57*<sup>CCS3\*</sup>, and the *hsNup57*<sup>CCS3\*</sup>•*hsNup49*<sup>CCS2+3\*</sup> complexes with 2:1 and 2:2 stoichiometry are shown from left to right.





**Fig. S23.**

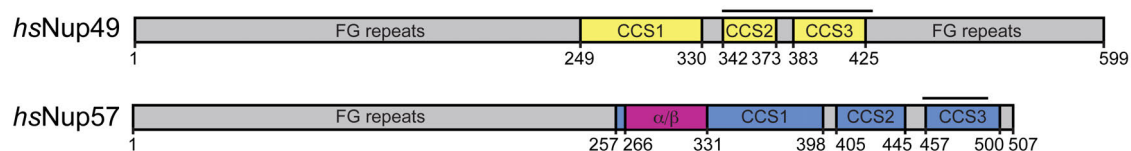
**Biochemical and structural analysis of the Nup57<sup>CCS3\*</sup> segment from different species.** (A) Domain architectures of the *C. thermophilum*, *H. sapiens*, and *R. norvegicus* Nup57 are shown and colored according to [Figure S19](#). SEC-MALS profiles of (B) Nup57<sup>CCS3\*</sup> and (C) hsNup57<sup>CCS3\*</sup>. Measured molecular masses are indicated for the peak fractions. Gray bars indicate fractions that were resolved on SDS-PAGE gels and visualized by Coomassie staining. (D) Structures of human and *C. thermophilum* Nup57<sup>CCS3\*</sup> shown in cartoon representation, shown in the same orientations as the previously determined rat Nup57<sup>CCS3\*</sup> (25). Two different states of hsNup57<sup>CCS3\*</sup> were obtained in different crystal packing arrangements. (E) Structural comparison of the *R. norvegicus* and *C. thermophilum* Nup57<sup>CCS3\*</sup> homo-tetramers. The Nup57<sup>CCS3\*</sup> and hsNup57<sup>CCS3\*</sup> homo-tetramers possess different helical packing arrangements and fail to superpose. As a reference, two corresponding residues Q473 (red) and Y293 of the *C. thermophilum* and *R. norvegicus* homo-tetramers, respectively, are indicated, illustrating their distinct helical packing. Notably, the *C. thermophilum* Nup57<sup>CCS3\*</sup> is homo-dimeric in solution, whereas the human homologue forms a homo-tetramer.



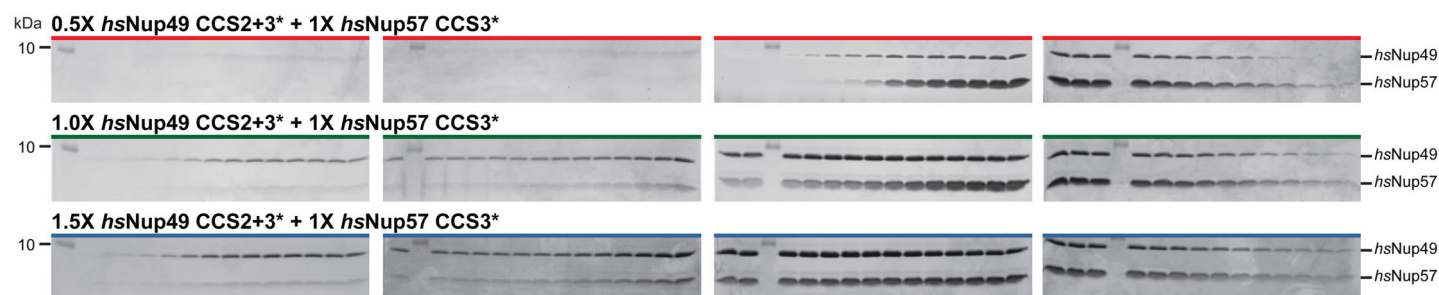
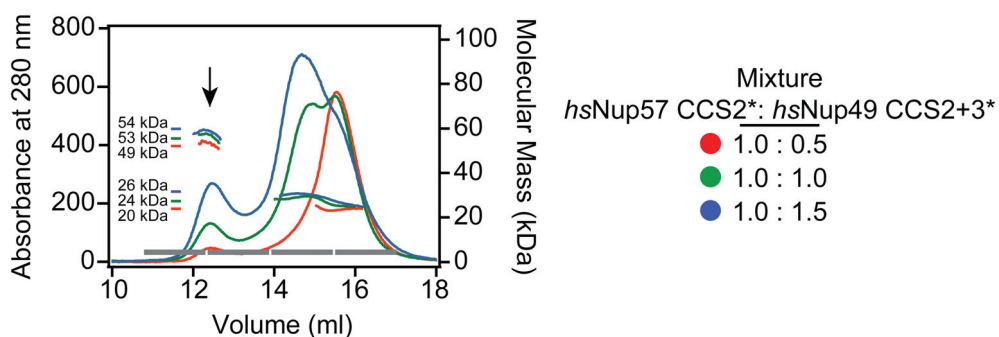
**Fig. S24.**

**Structure of *hsNup49*•*Nup57* with 2:2 stoichiometry.** Cartoon representation of the *hsNup57*<sup>CCS3\*</sup>•*hsNup49*<sup>CCS2+3\*</sup> hetero-dimer with 2:2 stoichiometry. Two orientations are shown from the front and side; *hsNup57*<sup>CCS3\*</sup> (blue) and *hsNup49*<sup>CCS2+3\*</sup> (yellow). A surface representation of the two views is shown on the right.

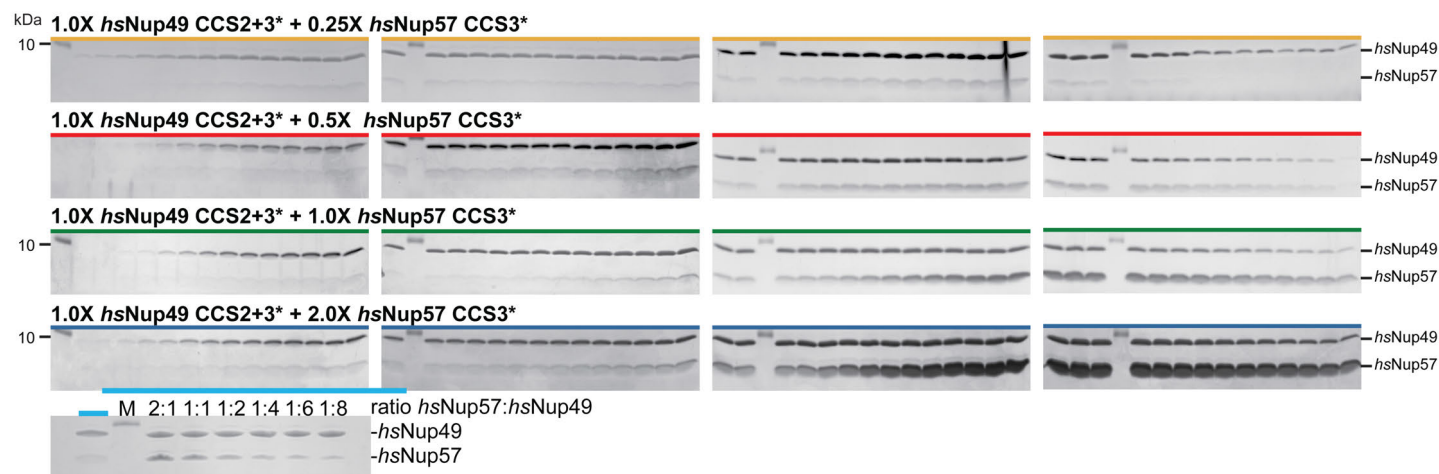
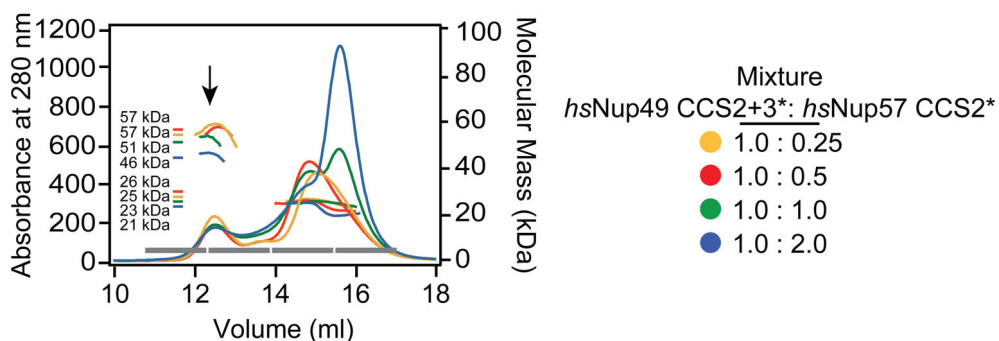
**A**



**B**

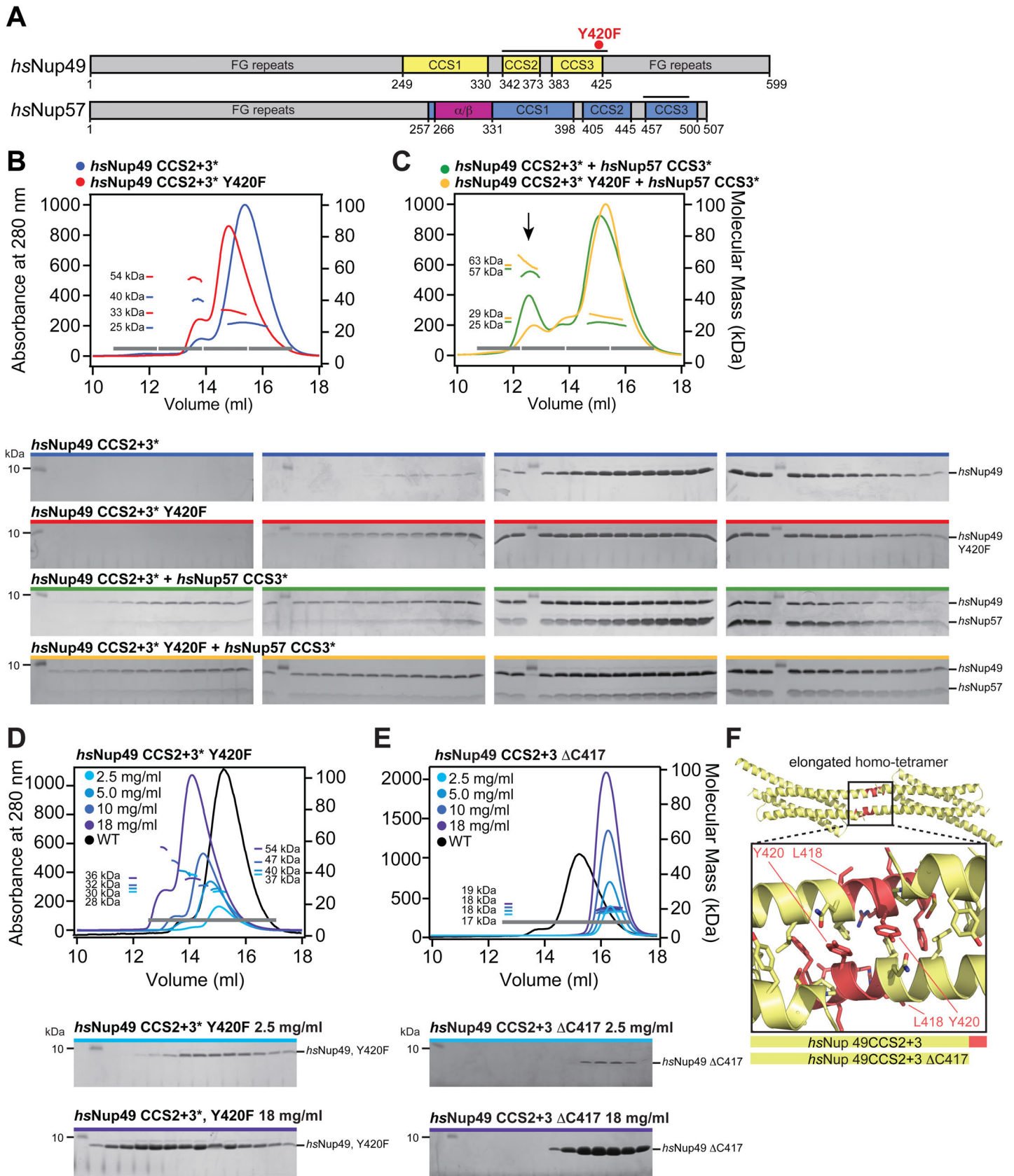


**C**



**Fig. S25.**

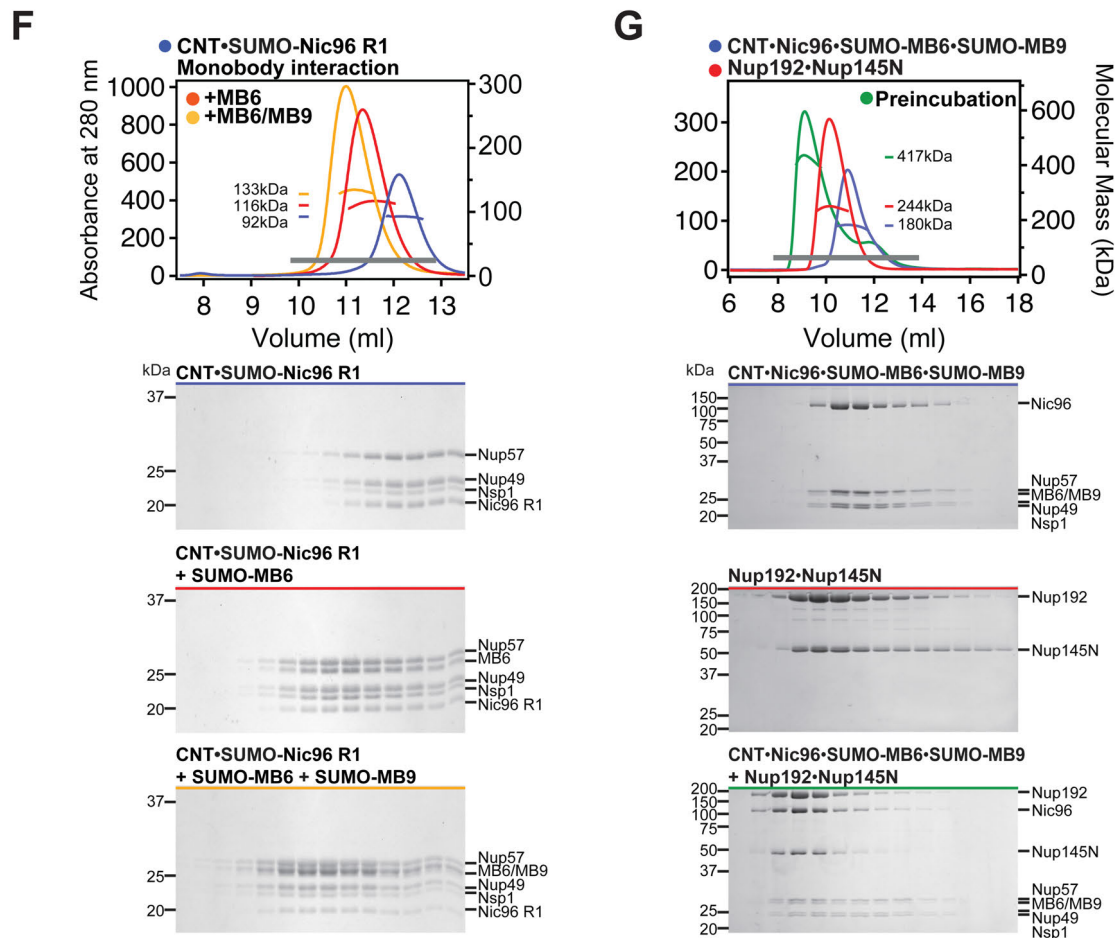
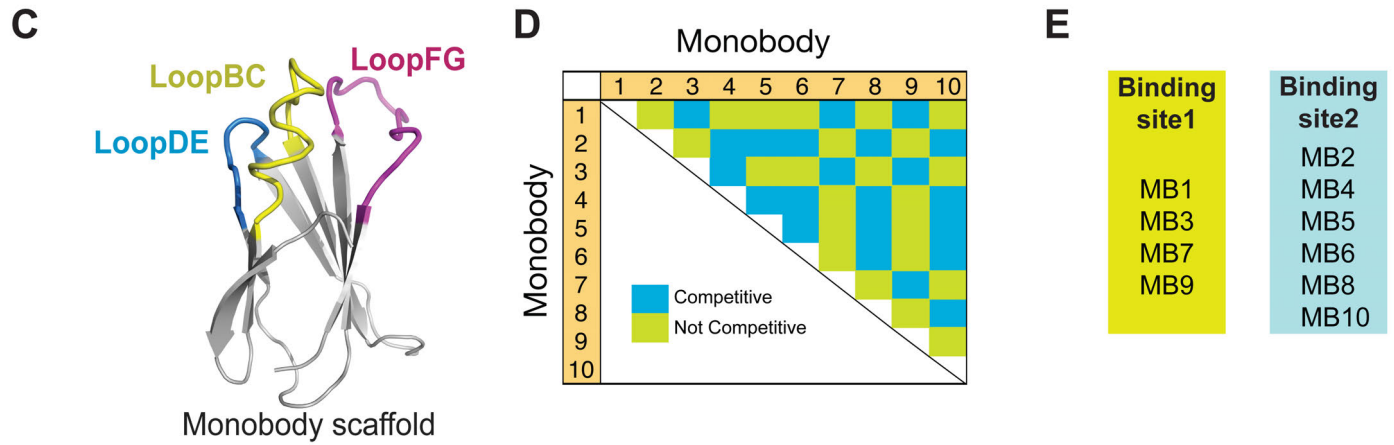
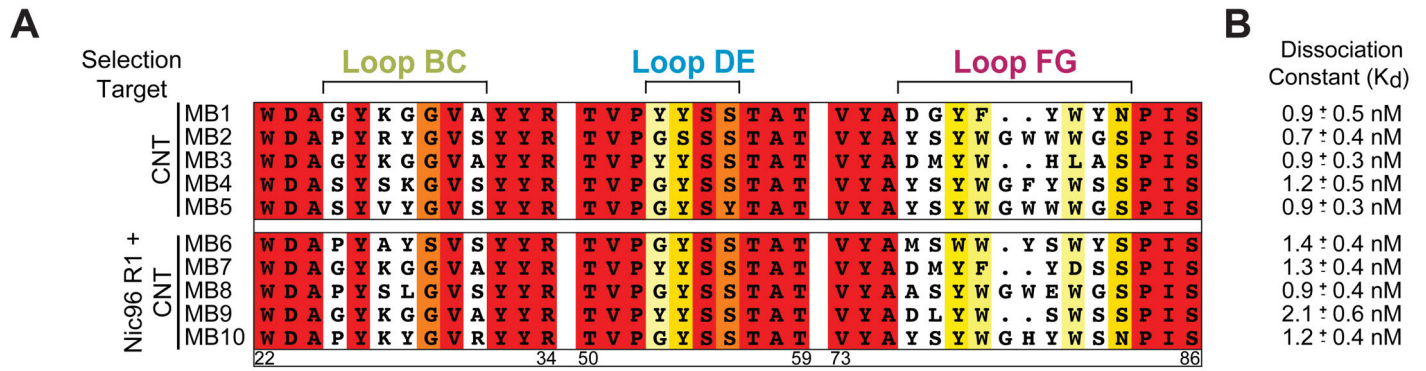
**Analysis of oligomeric state of human channel nup fragment mixtures.** (A) Domain architectures of the *H. sapiens* Nup49 and Nup57 are shown and colored according to [Figure S19](#). (B) SEC-MALS profiles of *hsNup57*<sup>CCS3\*</sup> preincubated with increasing molar amounts of *hsNup49*<sup>CCS2+3\*</sup> (red, green, and blue). (C) SEC-MALS profiles of *hsNup49*<sup>CCS2+3\*</sup> preincubated with increasing molar amounts of *hsNup57*<sup>CCS3\*</sup> (yellow, red, green, and blue). Measured molecular masses are indicated for the peak fractions. Gray bars indicate fractions that were resolved on SDS-PAGE gels and visualized by Coomassie staining. An arrow indicates the position of a higher molecular mass peak, which was previously described as a Nup57<sup>CCS3\*</sup>•Nup49<sup>CCS2+3\*</sup> complex with 6:4 stoichiometry, which constitutes the keystone for the open ring structure in the “ring model” (26). The abundance of this peak increases with increasing amounts of *hsNup49*<sup>CCS2+3\*</sup>, whereas it stays constant in the presence of increasing amounts of *hsNup57*<sup>CCS3\*</sup>. The corresponding SDS-PAGE analysis shows that Nup49 is substantially more abundant in this peak. To rule out that differential staining of the two proteins causes a perceived excess of *hsNup49*<sup>CCS2+3\*</sup>, *hsNup49*<sup>CCS2+3\*</sup> was mixed with different molar amounts of *hsNup57*<sup>CCS3\*</sup>, analyzed by SDS-PAGE and compared to the protein in the unknown peak (cyan). This analysis confirms an abundance of *hsNup49*<sup>CCS2+3\*</sup> in the peak.



**Fig. S26.**

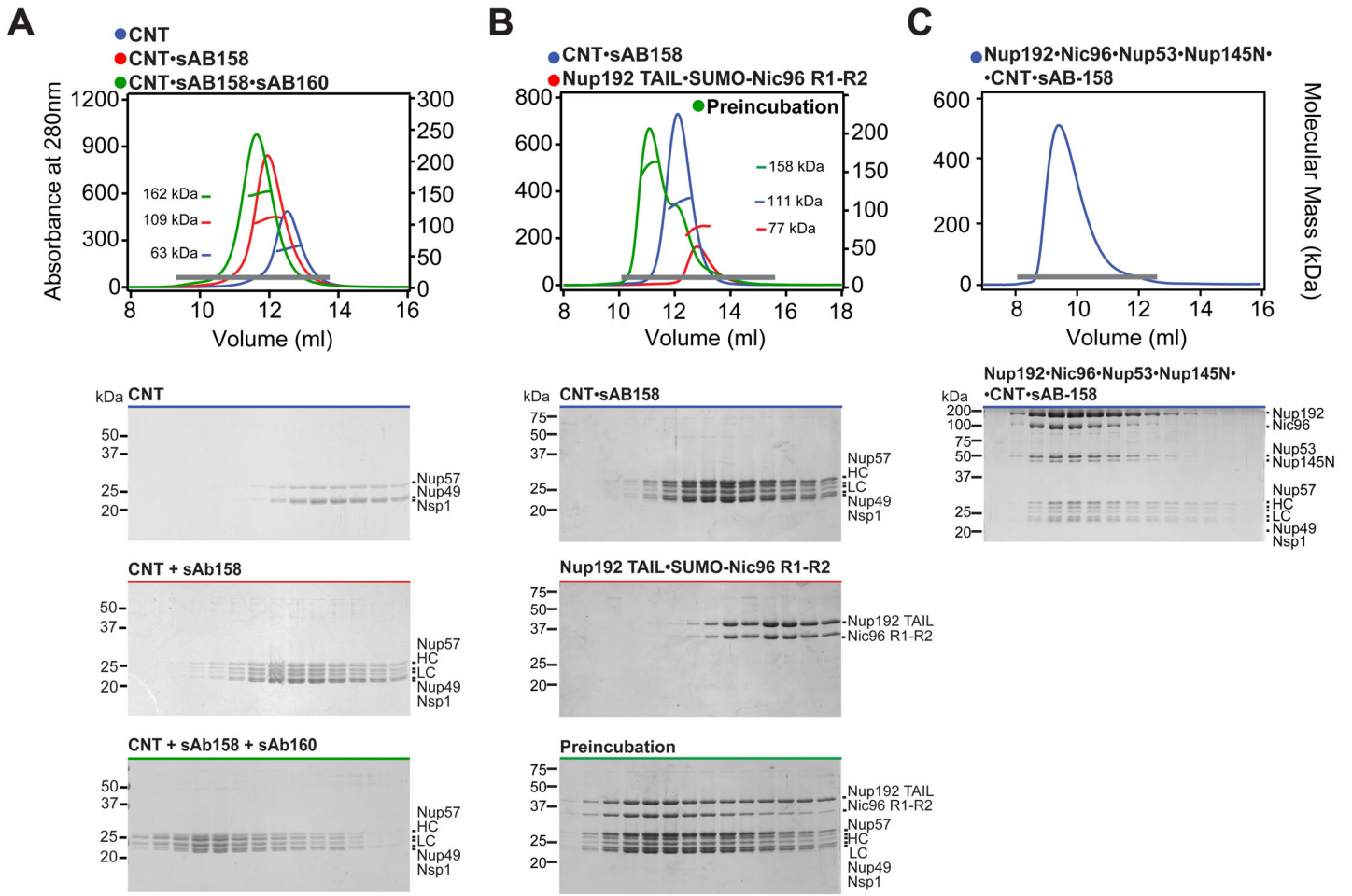
**Analysis of *hsNup49*<sup>CCS2+3\*</sup> mutants that affect the formation of higher oligomeric states.** (A) Domain architectures of the *H. sapiens* Nup49 and Nup57 are shown and colored according to Figure S19. A red dot indicates the position of the Y420F mutant in *hsNup49*, which was previously suggested to stabilize a *hsNup57*<sup>CCS3\*</sup>•*hsNup49*<sup>CCS2+3\*</sup> assembly with 6:4 stoichiometry (26). (B) SEC-MALS profiles of wild type *hsNup49*<sup>CCS2+3\*</sup> (blue) and *hsNup49*<sup>CCS2+3\*</sup> Y420F (red) in isolation and (C) preincubated with equimolar amounts of *hsNup57*<sup>CCS3\*</sup> (green and yellow; right). The *Nup49*<sup>CCS3\*</sup> Y420F mutant decreases the height of the ~60 kDa peak (black arrow) in the presence of *Nup57*<sup>CCS3\*</sup>, contrary to a previous report (26). SEC-MALS profiles of (D) *hsNup49*<sup>CCS2+3\*</sup> Y420F and (E) *hsNup49*<sup>CCS2+3\*</sup> ΔC417 at increasing protein concentrations. As reference, the wild type *hsNup49*<sup>CCS2+3\*</sup> SEC profile at a protein concentration of 18 mg/ml is shown (black). Measured molecular masses are indicated for the peak fractions. Gray bars indicate fractions that were resolved on SDS-PAGE gels and visualized by Coomassie staining. (F) Close-up view of the elongated hetero-tetramer interface of *hsNup49*<sup>CCS2+3\*</sup> in cartoon representation. The C-terminal two helical turns that are absent in the *hsNup49*<sup>CCS2+3\*</sup> ΔC417 mutant are colored in red. The *hsNup49*<sup>CCS2+3\*</sup> Y420F mutant forms higher order homo-oligomers with increasing molecular masses which is abolished in the *hsNup49*<sup>CCS2+3\*</sup> ΔC417 mutant.





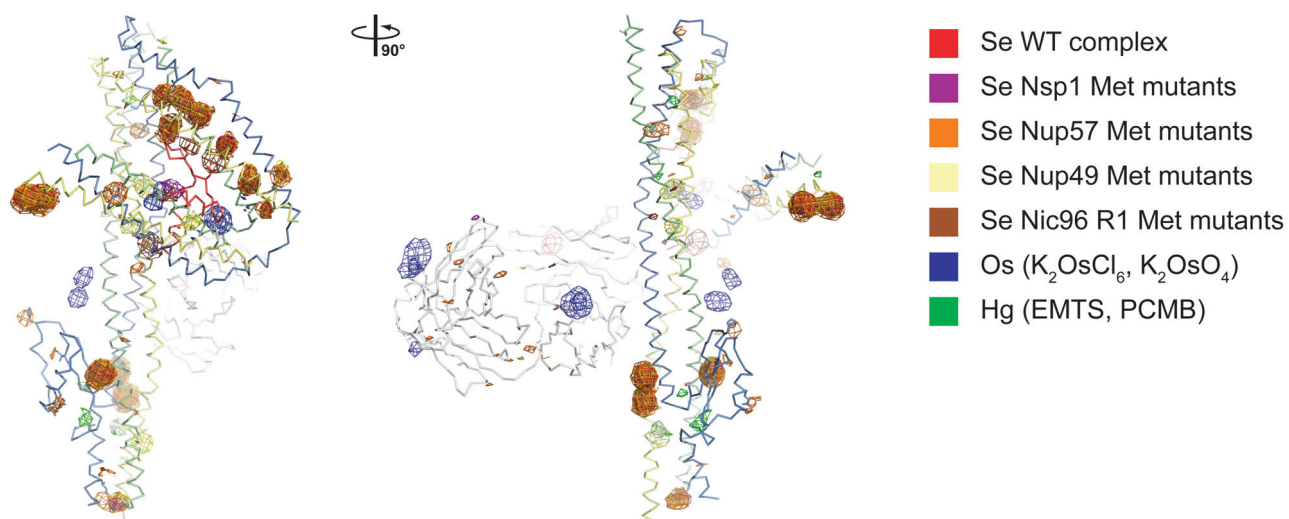
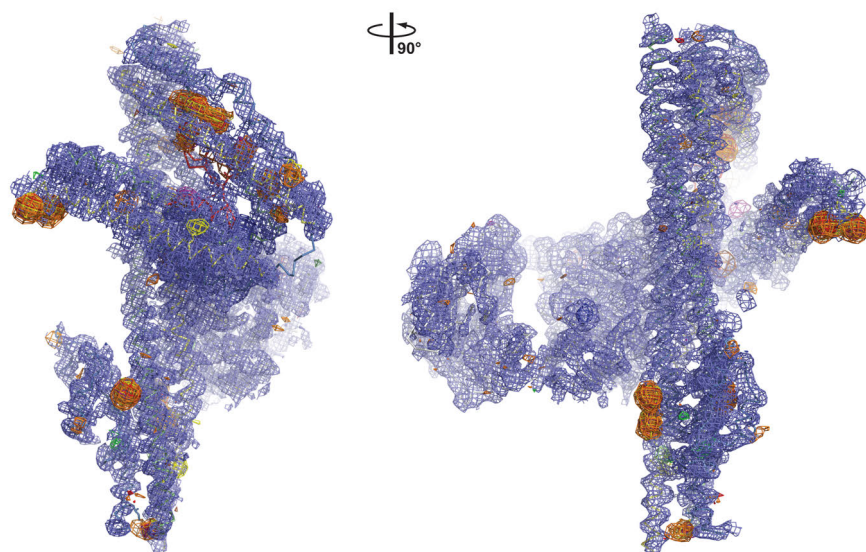
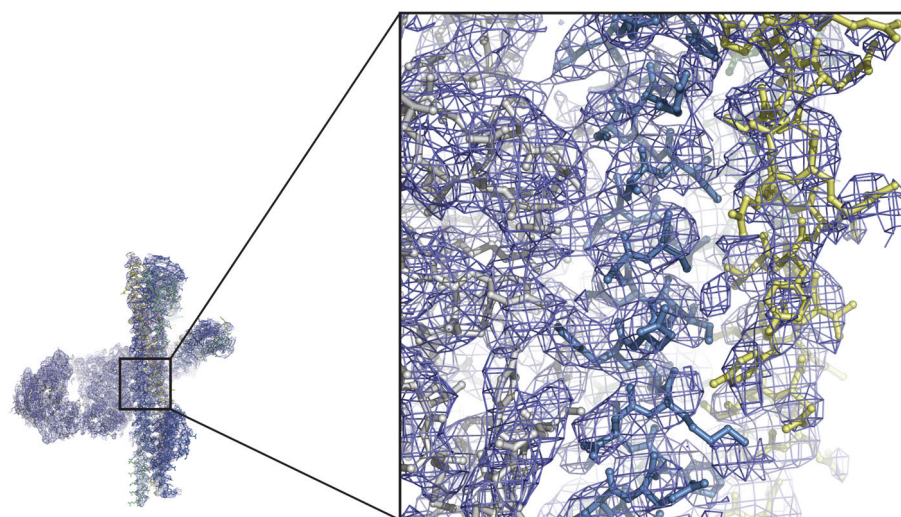
**Fig. S27.**

**Probing the CNT accessibility and assembly state in the IRC with synthetic monobodies.** (A) Sequence alignment of the variable loops (BC, DE, FG) of 10 monobodies generated against either the CNT or the CNT•Nic96<sup>R1</sup> complex. White bars indicate gaps in the alignment between loops. Single residue similarity is shaded from white (less than 40 % similarity), to yellow (40 % similarity), to red (100 % identity) using the BLOSUM62 matrix. (B) The dissociation constant of each monobody for CNT binding is shown. (C) The monobody scaffold is shown in cartoon representation highlighting the variable DE (blue), BC (yellow) and FG (magenta) loops. (D) A pairwise protein interaction matrix indicating competitive (blue) and non-competitive (green) binding to the CNT for the tested monobodies as determined by SEC-MALS analysis. (E) The monobodies are grouped according to their competitive CNT binding properties, indicating two distinct binding sites. (F) SEC-MALS profiles of CNT•SUMO-Nic96<sup>R1</sup> (blue), CNT•SUMO-Nic96<sup>R1</sup> bound to a single monobody (SUMO-MB6; red), and bound to 2 monobodies (SUMO-MB6 and SUMO-MB9; yellow) are shown. (G) SEC-MALS profiles of CNT•Nic96•SUMO-MB6•SUMO-MB9 (blue), Nup192•Nup145N (red), and after their preincubation (green) are shown. Measured molecular masses are indicated for the peak fractions. Gray bars indicate fractions that were resolved on SDS-PAGE gels and visualized by Coomassie staining. MALS and SDS-PAGE confirm the presence of the two monobodies MB6 and MB10 in the peak fractions of the IRC, confirming the steric accessibility of the CNT for monobody binding in solution.



**Fig. S28.**

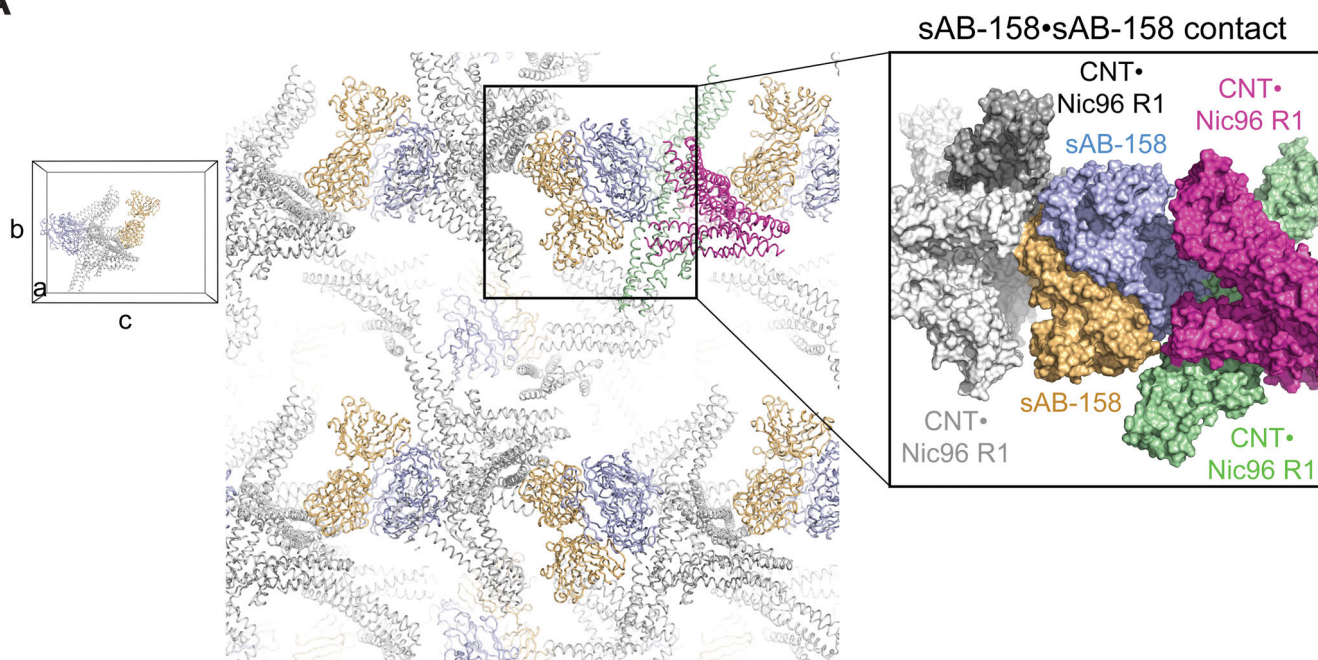
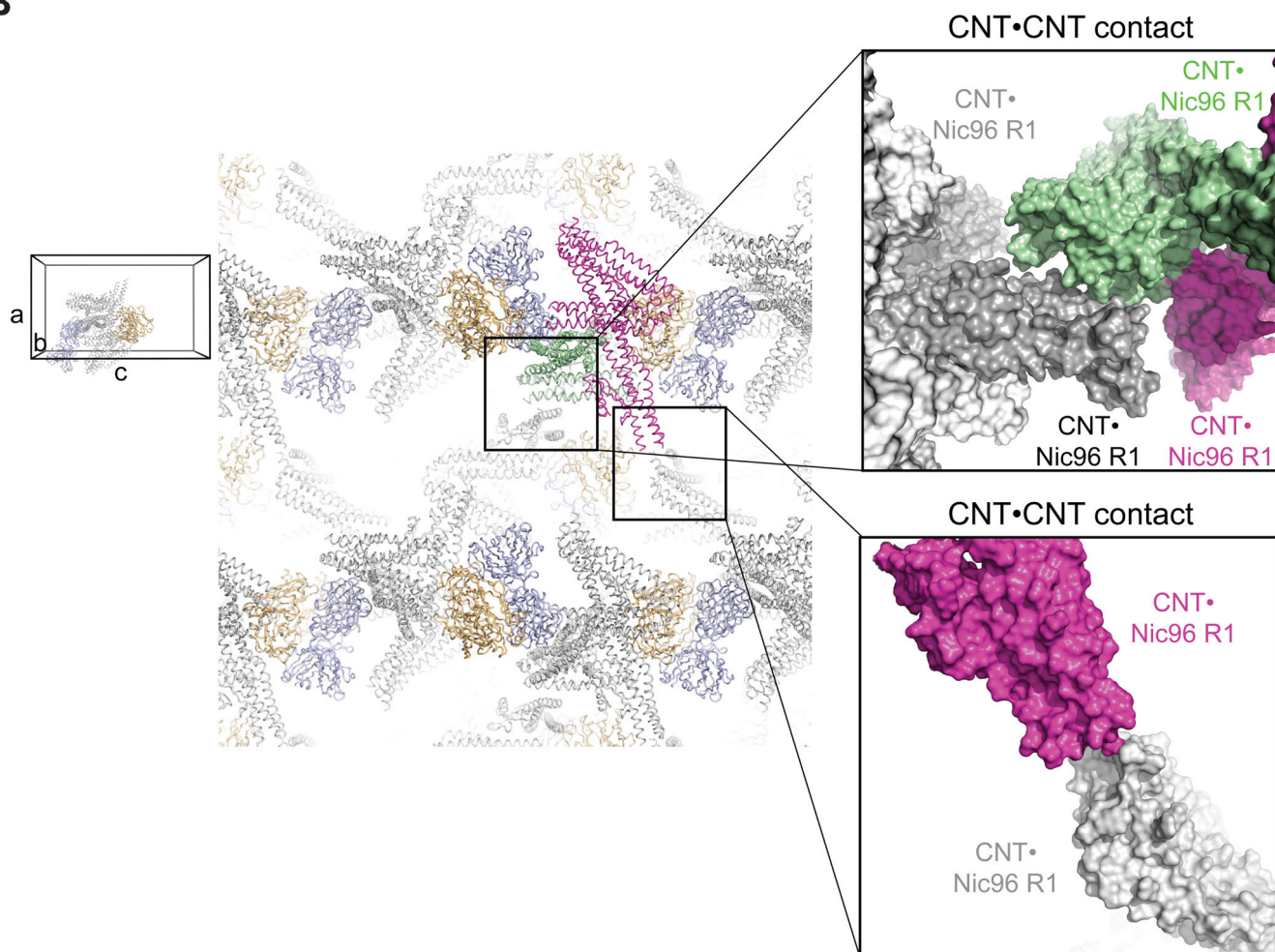
**Probing the CNT accessibility and assembly state in the IRC with synthetic antibodies.** (A) SEC-MALS profiles of the CNT (blue), the CNT preincubated with sAB-158 (red) and the CNT preincubated with sAB-158 and sAB-160 (green) are shown. (B) SEC-MALS profiles of the CNT•sAB-158 (blue), the Nup192<sup>TAIL</sup>•SUMO-Nic96<sup>R1-R2</sup> (red) and after their preincubation (green) are shown. (C) SEC-MALS profile of Nup192•Nic96•Nup145N•CNT•sAB-158 (blue). Measured molecular masses are indicated for the peak fractions. Gray bars indicate fractions that were resolved on SDS-PAGE gels and visualized by Coomassie staining. MALS and SDS-PAGE confirm stoichiometric complexes and the presence of sAb-158 in the peak fractions of the minimal and intact IRC, indicating the accessibility of the CNT for synthetic antibody binding and confirming that the CNT adopts the same assembly state in the IRC as observed in the crystal structure.

**A****B****C**

**Fig. S29.**

**Experimental phasing of the CNT•Nic96<sup>R1</sup>•sAB-158 structure.** (A) Anomalous difference Fourier maps were calculated from X-ray diffraction data collected at the selenium, osmium and mercury peak wavelengths. A ribbon representation of the structure of the CNT•Nic96<sup>R1</sup>•sAB-158 complex with the indicated anomalous difference Fourier maps contoured at 3.5  $\sigma$  is shown. The 29 introduced methionine mutants in Nsp1, Nup49, Nup57, and Nic96<sup>R1</sup> yielded additional 22 selenium sites, validating the sequence register. (B) Experimental electron density map after density modification of the CNT•Nic96<sup>R1</sup>•sAB-158 complex phased with the anomalous signal from crystals soaked with potassium hexachloroosmate (K<sub>2</sub>OsCl<sub>6</sub>), contoured at 1.0  $\sigma$ . The anomalous difference Fourier maps of the various heavy metal derivatives (panel A) are overlaid and contoured at 3.5  $\sigma$ . (C) Close-up view of a representative section of the experimental electron density map from panel B overlaid with a stick representation of the final model.



**A****B**

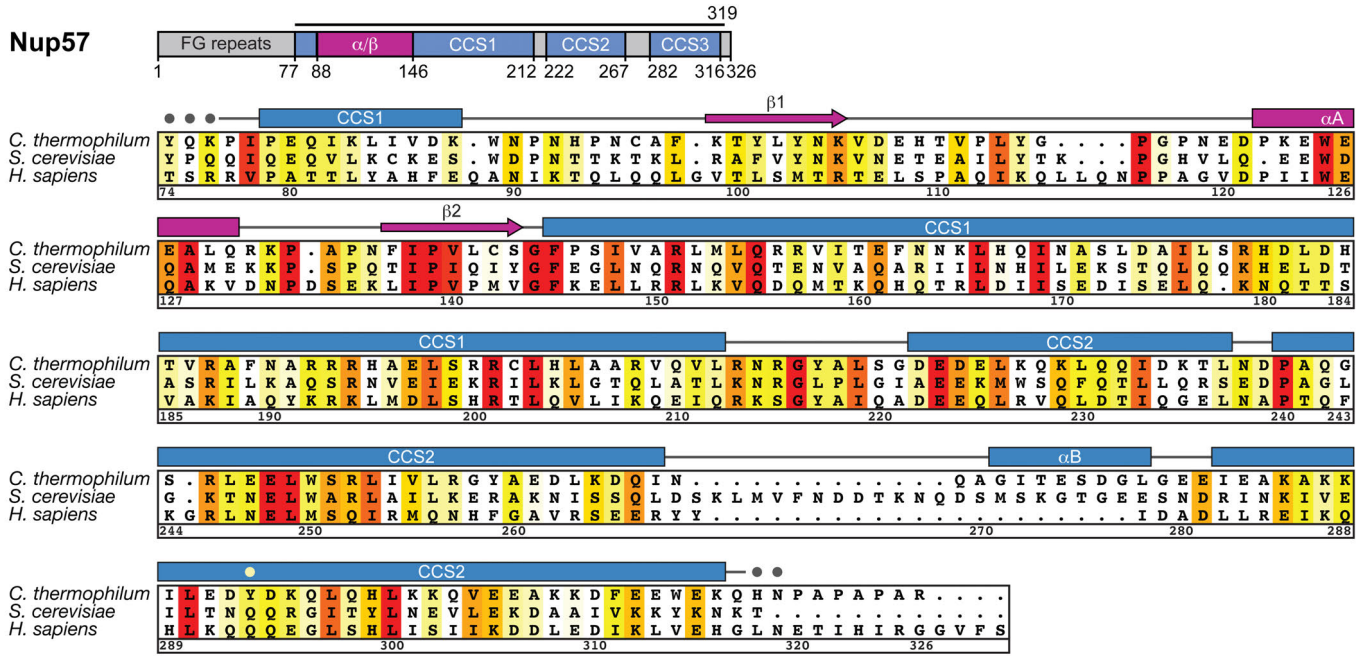


**Fig. S30.**

**Crystal packing interactions in the CNT•Nic96<sup>R1</sup>•sAB-158 crystals.** (A, B) Crystal packing interactions in the CNT•Nic96<sup>R1</sup>•sAB-158 crystal shown in two different orientations. Each asymmetric unit is composed of two CNT•Nic96<sup>R1</sup>•sAB-158 complexes that dimerize in a cross-handshake fashion. The major crystal contact is formed by an interaction between sAB-158 molecules (blue and orange) from adjacent asymmetric units along the c axis. Two additional minor crystal contacts are formed by CNT-CNT interactions along the a and b axes. CNT•Nic96<sup>R1</sup> complexes of one asymmetric unit are colored in green and magenta and their symmetry mates in light and dark gray. As references, units cell diagrams are shown to indicate the orientation of each view. The *Insets* mark the locations of the three crystal contact regions that are enlarged and shown in surface representation.

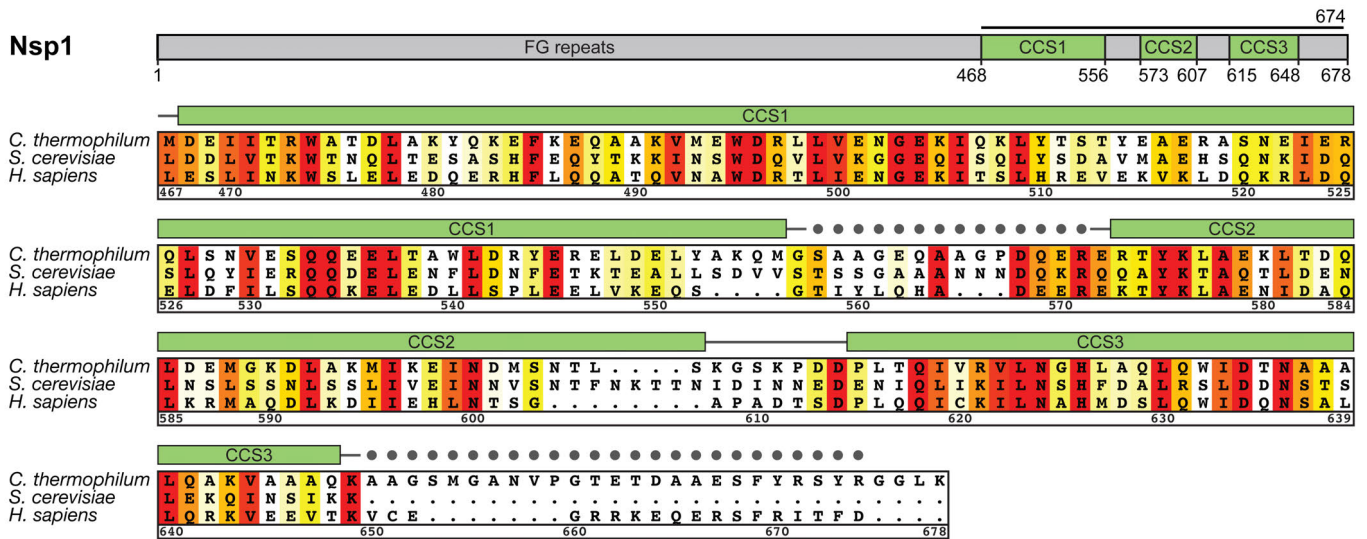
A

## Nup57



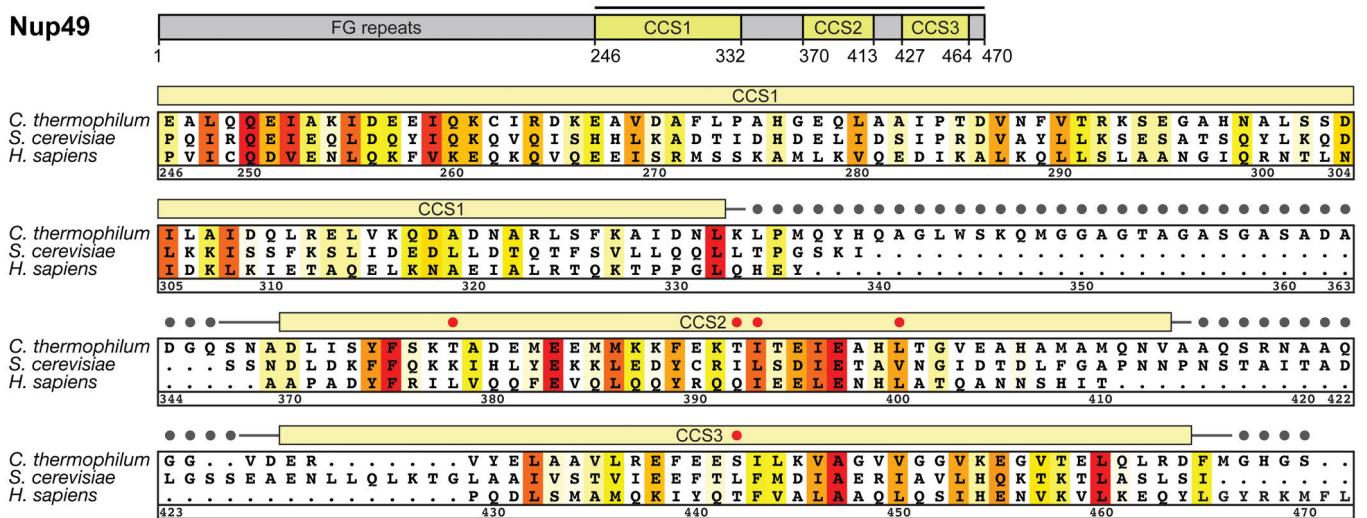
B

## Nsp1



C

## Nup49



Y420F

**Fig. S31.**

**Multispecies sequence alignment of the CNT coiled-coil segments.** Sequences of the three coiled-coil segments of (A) Nup57, (B) Nsp1 and (C) Nup49 from *C. thermophilum*, *S. cerevisiae*, and *H. sapiens* were aligned based on the secondary structure elements observed in the CNT•Nic96<sup>R1</sup> structure and colored by sequence similarity according to the BLOSUM62 (Blocks Substitution Matrix) matrix from white (less than 40 % similarity), to yellow (40 % similarity), to red (100 % identity). The numbering is according to the *C. thermophilum* channel nups. The secondary structure is indicated above the sequence as rectangles ( $\alpha$ -helices), arrows ( $\beta$ -strands), and lines (unstructured regions). Gray dots indicate residues with no observed electron density in the crystal structure, which are presumed disordered. Secondary structure elements are colored according to Figure 4A with the exception of the Nup57 $\alpha/\beta$  domain (magenta). A yellow dot in the secondary structure plot of Nup57 (A) indicates the position of Q473 and Y293, which are used as positional markers in the structural comparison of Nup57<sup>CCS3\*</sup> and hsNup57<sup>CCS3\*</sup> (Figure S23E). Residues of the EVPIP mutant are indicated by red dots in the secondary structure plot of Nup49 (panel C). A black dot and a black bar indicate the position of the Y420F mutant and the residues removed in the  $\Delta$ C417 mutant of hsNup49<sup>CCS2+3\*</sup>, which increases the abundance (Figure S26, A to C) and abolishes the formation (Figure S26, D and E) of higher homo-oligomeric states, respectively.

*C. thermophilum*  
*S. cerevisiae*  
*A. gossypii*  
*K. pastoris*  
*N. crassa*  
*A. nidulans*

. M F G F S G . . . . . S N N G . . . . . F G N K P A G S T G F S F G Q N N . . . . . N N T . . . . .  
 . M F N F G A N S G M N S N T G S T G G G L F G S T T N G G . G F S F G G N N N A S Q N N N T N V F S N T G T G V P P A G Q G T S N G G L F G M  
 M S F N F G A K . . . . . T N A A P . . . . . G N T S T G G . . . . . F S F G S N T S . . . . . N N A . . . . .  
 . . . . . . . . . . M S L F G G Q Q S G Q S L G L F S N T A K S S P F G T . . . . .

*C. thermophilum*  
*S. cerevisiae*  
*A. gossypii*  
*K. pastoris*  
*N. crassa*  
*A. nidulans*

N	T	T	P	S	A	S	G	F	G	.	.	.	.	F	G	G	S	S	Q	.	.	.	.	P	N	S	G	T	A	T	T	G	G	.	F	G	A	N	Q	A	T	N	T	.	.	F	G	S	N	Q	Q	S	.	S	T															
N	N	Q	T	T	N	T	G	I	G	G	N	S	G	L	F	G	G	A	A	N	T	Q	N	N	T	A	G	G	L	F	G	Q	T	Q	N	A	T	T	G	G	L	F	G	Q	N	T	Q	G	N	A	G	T	G	L	F	G	Q	N	T	Q	S	G	T	.	S	T				
A	P	A	A	G	S	S	S	S	F	S	.	.	.	S	G	L	F	G	A	K	P	.	T	Q	N	N	T	A	G	G	L	F	G	Q	T	Q	N	A	T	T	G	G	L	N	L	N	T	N	P	S	S	S	S	N	S	G	L	F	G	A	K	P	A	G	.	T	T	.	S	T

*C. thermophilum*  
*S. cerevisiae*  
*A. gossypii*  
*K. pastoris*  
*N. crassa*  
*A. nidulans*

[illegible]

*C. thermophilum*  
*S. cerevisiae*  
*A. gossypii*  
*K. pastoris*  
*N. crassa*  
*A. nidulans*

[illegible]

*C. thermophilum*  
*S. cerevisiae*  
*A. gossypii*  
*K. pastoris*  
*N. crassa*  
*A. nidulans*

[illegible]

*C. thermophilum*  
*S. cerevisiae*  
*A. gossypii*  
*K. pastoris*  
*N. crassa*  
*A. nidulans*

[illegible]

*C. thermophilum*  
*S. cerevisiae*  
*A. gossypii*  
*K. pastoris*  
*N. crassa*  
*A. nidulans*

Genomic map of the *B. subtilis* 168 genome showing the location of the CCS1 gene. The map displays the genome from 1 to 130 Mb. The CCS1 gene is located between approximately 80 Mb and 90 Mb. The map also shows the location of the  $\beta 1$  gene and the  $\alpha A$  gene. The CCS1 gene is flanked by the  $\beta 1$  gene and the  $\alpha A$  gene. The map shows the location of the CCS1 gene in the *B. subtilis* 168 genome.

*C. thermophilum*  
*S. cerevisiae*  
*A. gossypii*  
*K. pastoris*  
*N. crassa*  
*A. nidulans*

[illegible]

*C. thermophilum*  
*S. cerevisiae*  
*A. gossypii*  
*K. pastoris*  
*N. crassa*  
*A. nidulans*

Genomic map of the CCS1, CCS2, and CCS2 regions on chromosome 1. The map shows the locations of various SNPs and their corresponding alleles. The CCS1 region is on the left, followed by the CCS2 region, and then the CCS2 region on the right. The map is color-coded by SNP type: red for SNPs with a high frequency of the reference allele, yellow for SNPs with a moderate frequency, and green for SNPs with a low frequency. The map also shows the positions of the CCS1, CCS2, and CCS2 genes. The map is labeled with positions 200, 210, 220, 230, 240, 250, and 260.

*C. thermophilum*  
*S. cerevisiae*  
*A. gossypii*  
*K. pastoris*  
*N. crassa*  
*A. nidulans*

[illegible]

*C. thermophilum*  
*S. cerevisiae*  
*A. gossypii*  
*K. pastoris*  
*N. crassa*  
*A. nidulans*

Q	H	N	P	A	P	A	P	A	R
Y	K	N	K	T	.	.	.	.	.
Y	L	K	K	.	.	.	.	.	.
L	L	E	K	V	N	.	.	.	.
E	H	Q	P	T	P	A	P	M	.
S	L	S	.	.	.	.	.	.	.



**Fig. S32.**

**Multispecies sequence alignment of full-length Nup57.** Sequences from six diverse fungal species were aligned and colored by sequence similarity according to the BLOSUM62 matrix from white (less than 40 % similarity), to yellow (40 % similarity), to red (100 % identity). The numbering is according to *C. thermophilum* proteins. The secondary structure is indicated above the sequence as rectangles ( $\alpha$ -helices), arrows ( $\beta$ -strands), and lines (unstructured regions). Gray dots indicate residues with no observed electron density in the crystal structure, which are presumed disordered. Secondary structure elements of Nup57 are colored according to [Figure 4](#).

*C. thermophilum*  
*S. cerevisiae*  
*K. pastoris*  
*A. gossypii*  
*N. crassa*  
*A. nidulans*

[illegible]

*C. thermophilum*  
*S. cerevisiae*  
*K. pastoris*  
*A. gossypii*  
*N. crassa*  
*A. nidulans*

[illegible]

*C. thermophilum*  
*S. cerevisiae*  
*K. pastoris*  
*A. gossypii*  
*N. crassa*  
*A. nidulans*

[illegible]

*C. thermophilum*  
*S. cerevisiae*  
*K. pastoris*  
*A. gossypii*  
*N. crassa*  
*A. nidulans*

[illegible]

*C. thermophilum*  
*S. cerevisiae*  
*K. pastoris*  
*A. gossypii*  
*N. crassa*  
*A. nidulans*

G T S T S G S Q T T T P A K P L F S G S F G G S A T S P A G K A P . . . . . P T D A A A K T K E A G L K F G N L S K P A F S T G F G A K T S P T . . . . . K Q D G T A K .  
 T S N S G G . . . A T A S K P A L F S F F G A K S S S V D V Q Q S . . . . . S K P . . . A F S F G A A K Q S E E K K K D D N S A G . . . F S F G A K A K A D D . . . . .  
 S A S K D D D . . . A P K P A F S F F G A T Q P A S G N . . . . . L F G G A A A G G A E K K K S T P . . . T T T P A P S T A T S G G T T P P S . . . . .  
 N K P P A A D A S K A A P S L . L F G G A T Q P A S G N . . . . . L F G K T T T Q E S T P . . . . . S T A T S G T S G N . . . . .  
 A P S . . . . . L F G G A T Q P A S G N . . . . . L F K T T T Q E S T P . . . . . S T A T S G T S G N . . . . .

192 200 210 220 230 240 243

*C. thermophilum*  
*S. cerevisiae*  
*K. pastoris*  
*A. gossypii*  
*N. crassa*  
*A. nidulans*

[illegible]

*C. thermophilum*  
*S. cerevisiae*  
*K. pastoris*  
*A. gossypii*  
*N. crassa*  
*A. nidulans*

[illegible]

*C. thermophilum*  
*S. cerevisiae*  
*K. pastoris*  
*A. gossypii*  
*N. crassa*  
*A. nidulans*

[illegible]

*C. thermophilum*  
*S. cerevisiae*  
*K. pastoris*  
*A. gossypii*  
*N. crassa*  
*A. nidulans*

[illegible]

*C. thermophilum*  
*S. cerevisiae*  
*K. pastoris*  
*A. gossypii*  
*N. crassa*  
*A. nidulans*

[illegible]

*C. thermophilum*  
*S. cerevisiae*  
*K. pastoris*  
*A. gossypii*  
*N. crassa*  
*A. nidulans*

[illegible]

*C. thermophilum*  
*S. cerevisiae*  
*K. pastoris*  
*A. gossypii*  
*N. crassa*  
*A. nidulans*

[illegible]

*C. thermophilum*  
*S. cerevisiae*  
*K. pastoris*  
*A. gossypii*  
*N. crassa*  
*A. nidulans*

[illegible]

**Fig. S33.**

**Multispecies sequence alignment of full-length Nsp1.** Sequences from six species were aligned and colored by sequence similarity according to the BLOSUM62 matrix from white (less than 40 % similarity), to yellow (40 % similarity), to red (100 % identity). The numbering is according to *C. thermophilum* proteins. The secondary structure is indicated above the sequence as rectangles ( $\alpha$ -helices), arrows ( $\beta$ -strands), and lines (unstructured regions). Gray dots indicate residues with no observed electron density in the crystal structure, which are presumed disordered. Secondary structure elements of Nsp1 are colored according to [Figure 4](#).



*C. thermophilum*  
*S. cerevisiae*  
*A. gossypii*  
*K. pastoris*  
*A. nidulans*

*C. thermophilum*  
*S. cerevisiae*  
*A. gossypii*  
*K. pastoris*  
*A. nidulans*

*C. thermophilum*  
*S. cerevisiae*  
*A. gossypii*  
*K. pastoris*  
*A. nidulans*

*C. thermophilum*  
*S. cerevisiae*  
*A. gossypii*  
*K. pastoris*  
*A. nidulans*

*C. thermophilum*  
*S. cerevisiae*  
*A. gossypii*  
*K. pastoris*  
*A. nidulans*

*C. thermophilum*  
*S. cerevisiae*  
*A. gossypii*  
*K. pastoris*  
*A. nidulans*

*C. thermophilum*  
*S. cerevisiae*  
*A. gossypii*  
*K. pastoris*  
*A. nidulans*

*C.thermophilum*  
*S.cerevisiae*  
*A.gossypii*  
*K.pastoris*  
*A.nidulans*

*C. thermophilum*  
*S. cerevisiae*  
*A. gossypii*  
*K. pastoris*  
*A. nidulans*

G	H	G	S	.
.	.	.	.	.
P	V	I	S	S
.	.	.	.	.
G	L	R	Q	A
467	470			



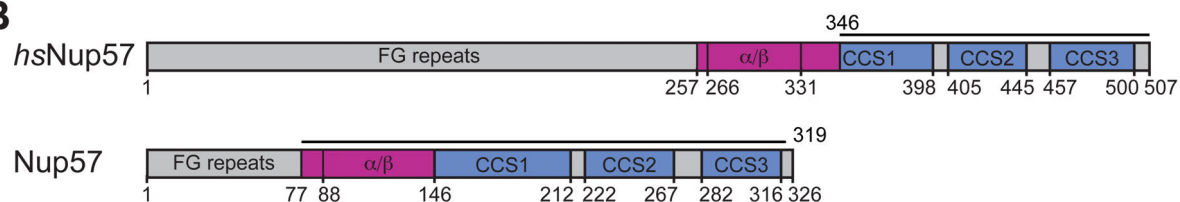
**Fig. S34.**

**Multispecies sequence alignment of full-length Nup49.** Sequences from six diverse fungal species were aligned and colored by sequence similarity according to the BLOSUM62 matrix from white (less than 40 % similarity), to yellow (40 % similarity), to red (100 % identity). The numbering is according to *C. thermophilum* proteins. The secondary structure is indicated above the sequence as rectangles ( $\alpha$ -helices), arrows ( $\beta$ -strands), and lines (unstructured regions). Gray dots indicate residues with no observed electron density in the crystal structure, which are presumed disordered. Secondary structure elements of Nup49 are colored according to [Figure 4](#).

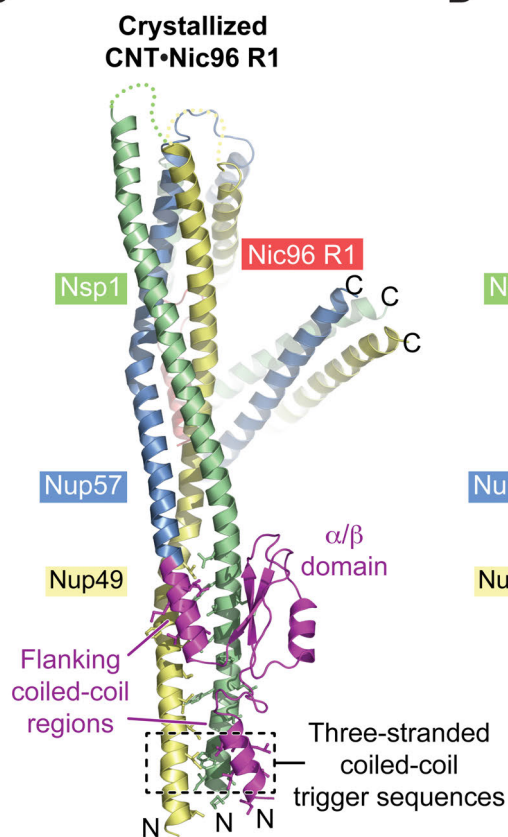
**A**

Nsp1	467	M	D	E	I	I	T	R	473
Nup49	249	Q	Q	E	I	A	K	I	255
Nup57	78	I	P	E	Q	I	K	L	84
consensus 1		(I/V/L)	X	(D/E)	I	X	(R/K)	X	
consensus 2		(I/V/L)	(D/E)	X	I	X	(R/K)	X	

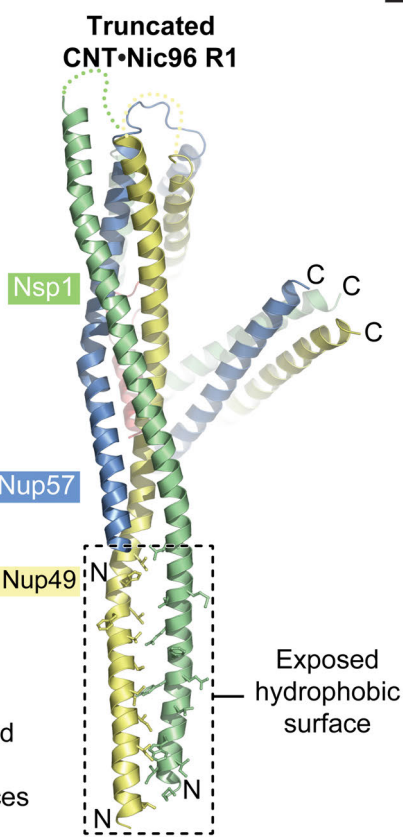
**B**



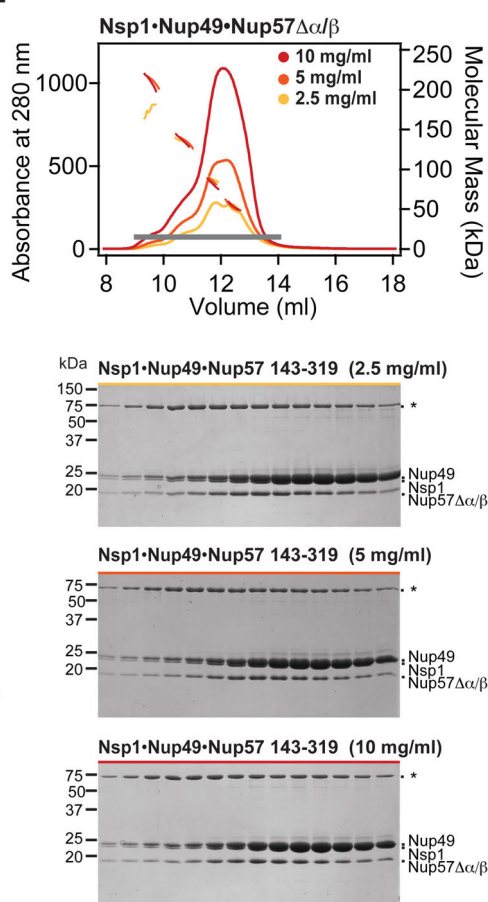
**C**



**D**

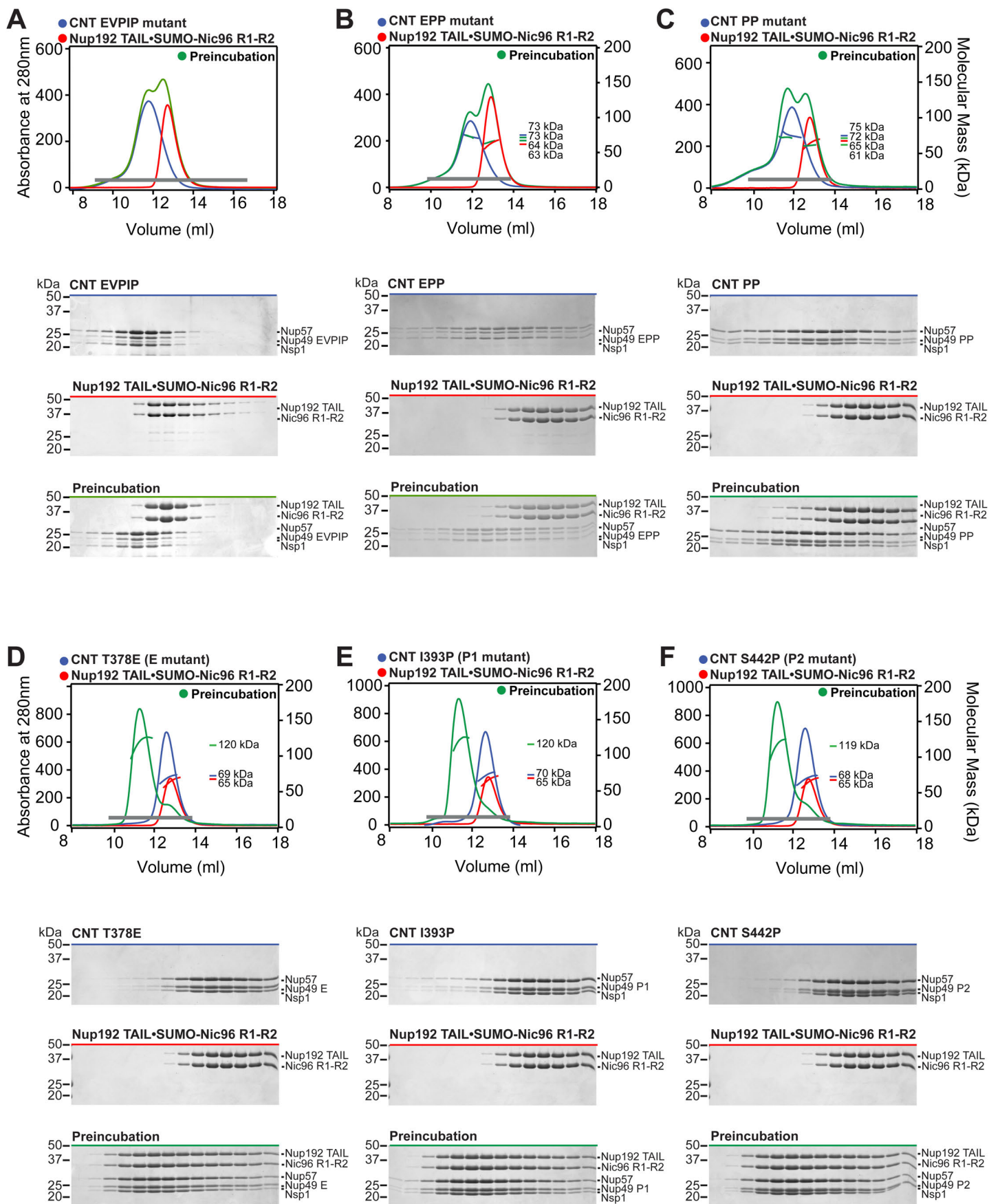


**E**



**Fig. S35.**

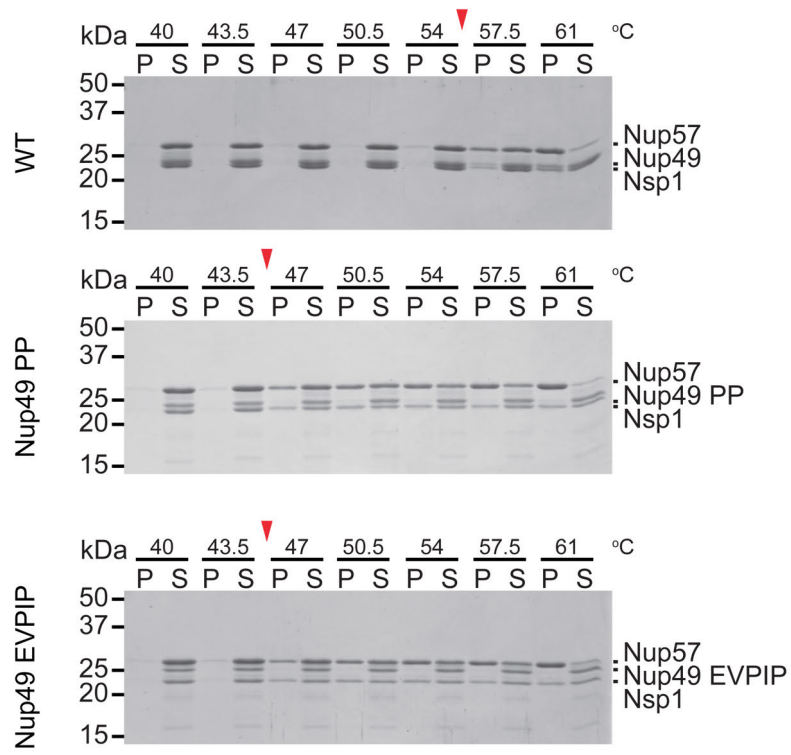
**Removal of the Nup57  $\alpha/\beta$  insertion domain results in non-stoichiometric higher-order CNT oligomers.** (A) Alignment of the putative three-stranded coiled-coil trigger sequences of Nsp1 (green), Nup49 (yellow) and Nup57 (blue) is shown. The consensus sequence is depicted in red (X, any residue) (36). (B) Domain structures of *H. sapiens* and *C. thermophilum* Nup57. Bars above the domain structures indicate the domain boundaries of intact Nup57 as observed in the CNT•Nic96<sup>R1</sup>•sAB-158 crystal structure and of *rn*Nup57 and *hs*Nup57 fragments that were reported previously to form various vastly different CNT oligomerization states in solution (22, 23, 26, 28). (C) CNT•Nic96<sup>R1</sup>•sAB-158 structure is shown in cartoon representation colored according to Figure 4. The Nup57  $\alpha/\beta$  insertion domain and flanking coiled-coil regions that were excluded in previous mammalian CNT reconstitutions are colored in magenta. Hydrophobic residues in the coiled-coil center are represented in ball-and-stick representation. The location of the putative three-stranded coiled-coil trigger sequences is highlighted on the bottom. For clarity, sAB-158 was removed from the illustration. (D) Model of the CNT lacking an N-terminal Nup57 region that encodes the  $\alpha/\beta$  insertion domain and flanking coiled-coil regions, corresponding to the mammalian Nup57 fragments used in previous studies. (E) SEC-MALS profiles of a *C. thermophilum* CNT variant using a Nup57 fragment that lacks the N-terminal coiled-coil region and the  $\alpha/\beta$  insertion domain (residues 146-319) at three different concentrations. Measured molecular masses are indicated for the peak fractions. Gray bar indicates fractions that were resolved on SDS-PAGE gels and visualized by Coomassie staining. Asterisks indicate degradation products or contaminations. MALS and SDS-PAGE analysis establish that the removal of the Nup57  $\alpha/\beta$  insertion domain results in a CNT that adopts various different oligomeric states in solution, as previously observed for the mammalian truncated CNT (22, 23, 26, 28).



**Fig. S36.**

**Identification of CNT mutations that disrupt binding to the IRC.** (A-F) SEC-MALS and SDS-PAGE analysis corresponding to [Figure 5](#). SEC-MALS profiles of mutant CNT or Nup192<sup>TAIL</sup>•SUMO-Nic96<sup>R1-R2</sup> complexes are shown individually (blue and red) and after their preincubation (green). Measured molecular masses are indicated for the peak fractions. Gray bars indicate fractions that were resolved on SDS-PAGE gels and visualized by Coomassie staining.

CNT

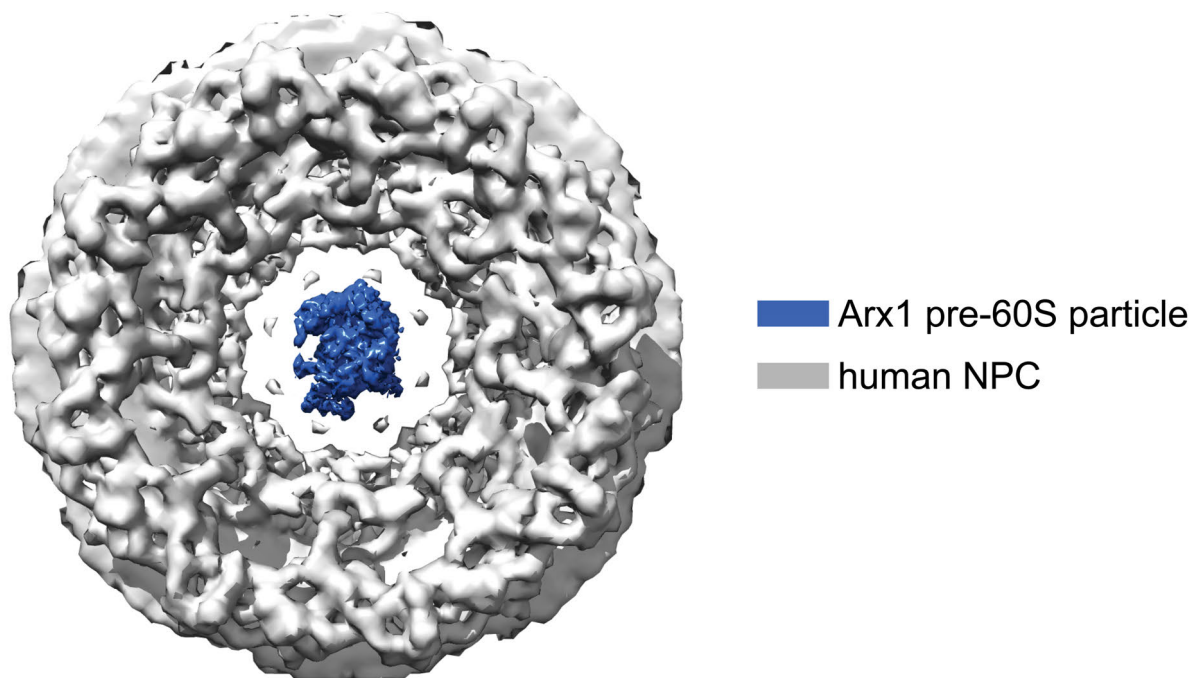


**Fig. S37.**

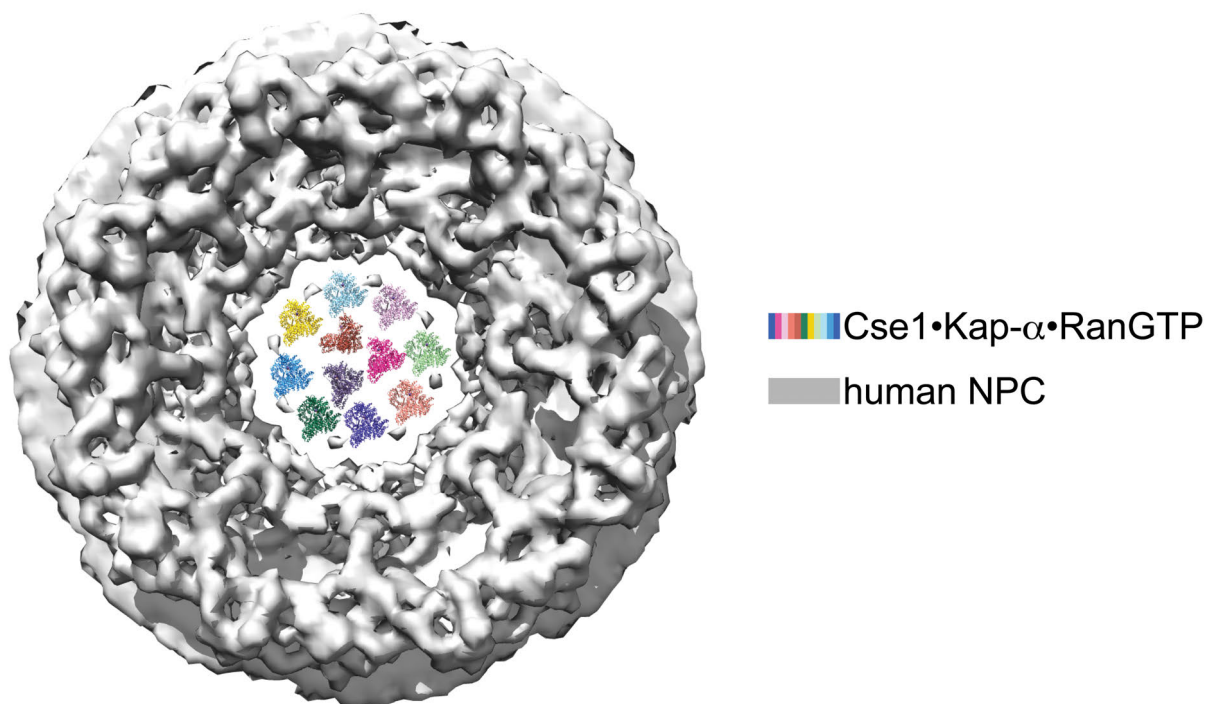
**Nup49 PP and EVPIP mutants decrease CNT thermostability.** Wild type CNT and CNT carrying either the Nup49 PP or EVPIP mutant were incubated at the indicated temperatures for 30 minutes prior to centrifugation. Soluble (S) and pelleted (P) fractions were analyzed by SDS-PAGE and visualized by Coomassie staining. Red arrows indicate temperature increment at which *in vitro* aggregation ensued.



A



B





**Fig. S38.**

**The human NPC can accommodate the 60S pre-ribosomal particle.** A top view of the cryoelectron tomography reconstruction of the human NPC is shown (EMD 2444; gray). The central transport channel is large enough to accommodate (**A**) the 60S pre-ribosomal particle (EMD 2528; blue) and (**B**) eleven Cse1•Kap- $\alpha$ •RanGTP nuclear export complexes (PDB ID: 1WA5) (2, 39, 65).

**Table S1.**

## SEC-MALS analysis

Figure	Nucleoporin or nucleoporin complex	Experimental Mass (kDa)	Theoretical Mass (kDa)	Stoichiometry
Fig. 1B Fig. S1A	CNT•Nic96	180	187	equimolar
	Nup192•Nup145N	250	239	equimolar
	Nup192•Nup145N •CNT•Nic96	425	426	equimolar
Fig. 1C Fig. S1B	CNT•Nic96•Nup192•Nup53	320	424	below measurement limit
	Nup82•SUMO-Nup159 <sup>T</sup> •Nup145N	110	123	equimolar
	Nup82•SUMO-Nup159 <sup>T</sup> •Nup145N•CNT•Nic96•Nup192•Nup53	440	547	below measurement limit
Fig. 1D Fig. S1C	CNT•Nup192•Nup145N•SUMO-Nic96 <sup>R1-R2</sup>	291	345	below measurement limit
	Nic96 <sup>SOL</sup>	75	81	n/a
	CNT•Nup192•Nup145N•SUMO-Nic96 <sup>R1-R2</sup> •Nic96 <sup>SOL</sup>	301, 74	426	no interaction
Fig. 1E Fig. S1D	CNT	74	77	equimolar
	Nup192 <sup>TAIL</sup> •SUMO-Nic96 <sup>R1-R2</sup>	69	72	equimolar
	Nup192 <sup>TAIL</sup> •SUMO-Nic96 <sup>R1-R2</sup> •CNT	135	149	equimolar
Fig. 1F Fig. S1E	CNT	74	77	equimolar
	SUMO-Nic96 <sup>R1</sup>	18	18	n/a
	CNT•SUMO-Nic96 <sup>R1</sup>	87, 18	95	equimolar
Fig. 1G Fig. S1F	Nup192 <sup>TAIL</sup>	39	41	n/a
	SUMO-Nic96 <sup>R2</sup>	18	19	n/a
	Nup192 <sup>TAIL</sup> •SUMO-Nic96 <sup>R2</sup>	54, 19	60	equimolar
Fig. 1H Fig. S1G	CNT	68	77	equimolar
	Nup192•Nup145N•Nic96 <sup>R2-SOL</sup>	358	336	equimolar
	Nup192•Nup145N•Nic96 <sup>R2-SOL</sup> •CNT	360, 73	413	no interaction
Fig. 1I Fig. S1H	CNT	70	77	equimolar
	Nup192•Nup53•Nic96 <sup>R2-SOL</sup>	355	333	equimolar
	Nup192•Nup53•Nic96 <sup>R2-SOL</sup> •CNT	350, 70	410	no interaction
Fig. S2A	Nup82 <sup>NTD</sup> •SUMO-Nup159 <sup>T</sup>	79	80	equimolar
	Nup145N	42	42	n/a
	Nup82 <sup>NTD</sup> •SUMO-Nup159 <sup>T</sup> •Nup145N	110	122	equimolar
Fig. S2B	Nup82 <sup>NTD</sup> •SUMO-Nup159 <sup>T</sup>	79	80	equimolar
	Nup145N <sup>APD</sup>	16	16	n/a
	Nup82 <sup>NTD</sup> •SUMO-Nup159 <sup>T</sup> •Nup145N <sup>APD</sup>	97	96	equimolar
Fig. S2D	CNT	71	77	equimolar
	Nup145N	48	42	n/a
	CNT•Nup145N	105	119	equimolar
Fig. S2F	CNT•Nup145N	78	119	equimolar
	Nup192	182	197	n/a
	Nup192•CNT•Nup145N	210, 76	316	no interaction
Fig. S4A	CNT	74	77	equimolar
	SUMO-Nic96 <sup>R2</sup>	18	19	n/a
	CNT•SUMO-Nic96 <sup>R2</sup>	75, 18	96	No interaction
Fig. S4B	Nup192 <sup>TAIL</sup>	39	41	n/a
	SUMO-Nic96 <sup>R1</sup>	18	18	n/a
	Nup192 <sup>TAIL</sup> •SUMO-Nic96 <sup>R1</sup>	29	59	No interaction
Fig. S4C	CNT	74	77	equimolar
	Nup192 <sup>TAIL</sup>	39	41	n/a
	CNT•Nup192 <sup>TAIL</sup>	74, 40	118	No interaction
Fig. 3B Fig. S15B	CNT	71	77	equimolar
	SUMO-Nic96 <sup>R1</sup>	18	18	n/a
	Preincubation - LLLL	18, 70	95	no interaction
Fig. S15A	Preincubation - wt	17, 80	95	equimolar
	CNT	71	77	equimolar
	SUMO-Nic96 <sup>R1</sup> wt and truncations	18	18, 17, 18, 17	n/a
	Preincubation - wt	17, 80	95	equimolar
	Preincubation - truncations	18, 18, 18, 73, 70, 68	94-95	sub-stoichiometric / no interaction
Fig. S18C	CNT 1 mg/ml	147, 71	77 for monomer	equimolar
	CNT 10 mg/ml	131, 69	77 for monomer	equimolar
	CNT 30 mg/ml	136, 73	77 for monomer	equimolar

Figure	Nucleoporin or nucleoporin complex	Experimental Mass (kDa)	Theoretical Mass (kDa)	Stoichiometry
Fig. S18D	CNT	147, 71	77 for monomer	equimolar
	SUMO-Nic96 <sup>R1</sup>	---	18	n/a
	CNT•SUMO-Nic96 <sup>R1</sup>	183, 87	95 for monomer	equimolar
Fig. S15A	CNT	71	77	equimolar
	SUMO-Nic96 <sup>R1</sup> wt and truncations	18	18, 17, 18, 17	n/a
	Preincubation - wt	17, 80	95	equimolar
	Preincubation - truncations	18, 18, 18, 73, 70, 68	94-95	sub-stoichiometric / no interaction
	hsNup49 CCS2+3*	22, 23, 25, 29, 33, 36, 37, 40	10 for monomer	n/a
Fig. S22B	hsNup49 CCS2+3*	22, 23, 25, 29, 33, 36, 37, 40	10 for monomer	n/a
	hsNup57 CCS3*	18	5 for monomer	n/a
	hsNup49 CCS2+3*	25, 40	10 for monomer	n/a
Fig. S22C	hsNup57 CCS3*	18	5 for monomer	n/a
	hsNup49 CCS2+3*	25, 40	10 for monomer	n/a
	hsNup57 CCS3*•hsNup49 CCS2+3*	24, 57	various	polydisperse
Fig. S23B	hsNup57 CCS3*	18	5 for monomer	tetramer
Fig. S23C	Nup57 CCS3*	8	4 for monomer	dimer
Fig. S25B	hsNup57 CCS3*	---	5 for monomer	n/a
	hsNup49 CCS2+3*	---	10 for monomer	n/a
	hsNup57 CCS3*•hsNup49 CCS2+3*	20, 24, 26, 49, 53, 54	various	polydisperse
Fig. S25C	hsNup57 CCS3*	---	5 for monomer	n/a
	hsNup49 CCS2+3*	---	10 for monomer	n/a
	Preincubation	21, 23, 25, 26, 46, 51, 57, 57	various	polydisperse
Fig. S26B	hsNup57 CCS3*	---	5 for monomer	n/a
	hsNup49 CCS2+3*	---	10 for monomer	n/a
	hsNup49 CCS2+3*, Y420F	---	10 for monomer	n/a
	hsNup57 CCS3*•hsNup49 CCS2+3*	25, 57	various	polydisperse
	hsNup57 CCS3*•hsNup49 CCS2+3*, Y420F	29, 63	various	polydisperse
Fig. S26C	hsNup49 CCS2+3*	25, 40	10 for monomer	polydisperse
	hsNup49 CCS2+3*, Y420F	33, 54	10 for monomer	polydisperse
Fig. S26D	hsNup49 CCS2+3*, Y420F	28, 30, 32, 36, 37, 40, 47, 54	10 for monomer	polydisperse
Fig. S26E	hsNup49 CCS2+3* ΔC417	17, 18, 18, 19	10 for monomer	polydisperse
Fig. S27F	CNT•SUMO-Nic96 <sup>R1</sup>	92	95	n/a
	CNT•SUMO-Nic96 <sup>R1</sup> •MB6	116	119	equimolar
	CNT•SUMO-Nic96 <sup>R1</sup> •MB6•MB9	133	143	equimolar
Fig. S27G	CNT•Nic96•SUMO-MB6•SUMO-MB9	180	236	equimolar
	Nup192•Nup145N	244	239	equimolar
	CNT•Nic96•SUMO-MB6•SUMO-MB9•Nup192•Nup145N	417	475	equimolar
Fig. S28A	CNT	63	77	equimolar
	CNT•sAB158	109	128	equimolar
	CNT•sAB158•sAB160	162	179	equimolar
Fig. S28B	CNT•sAB158	111	128	equimolar
	Nup192 <sup>TAIL</sup> •SUMO-Nic96 <sup>R1-R2</sup>	77	72	equimolar
	CNT•sAB158•Nup192 <sup>TAIL</sup> •SUMO-Nic96 <sup>R1-R2</sup>	158	200	equimolar
Fig. 5C Fig. S36B	CNT EPP	73	77	equimolar
	Nup192 <sup>TAIL</sup> •SUMO-Nic96 <sup>R1-R2</sup>	63	72	equimolar
	Preincubation - wt	135	149	equimolar
Fig. S36C	Preincubation – EPP mutant	73, 64	149	no interaction
	CNT PP	75	77	equimolar
	Nup192 <sup>TAIL</sup> •SUMO-Nic96 <sup>R1-R2</sup>	65	72	equimolar
Fig. S36D	Preincubation	72, 61	149	below measurement limit
	CNT T378E	69	77	equimolar
	Nup192 <sup>TAIL</sup> •SUMO-Nic96 <sup>R1-R2</sup>	65	72	equimolar
Fig. S36E	Preincubation	120	149	equimolar
	CNT I393P	70	77	equimolar
	Nup192 <sup>TAIL</sup> •SUMO-Nic96 <sup>R1-R2</sup>	65	72	equimolar
Fig. S36F	Preincubation	120	149	equimolar
	CNT S442P	68	77	equimolar
	Nup192 <sup>TAIL</sup> •SUMO-Nic96 <sup>R1-R2</sup>	65	72	equimolar
	Preincubation	119	149	equimolar

**Table S2.**Crystallographic analysis of Nup188<sup>TAIL</sup>, Nup192<sup>TAIL</sup>, and Nup82<sup>NTD</sup>•Nup159<sup>T</sup>•Nup145N<sup>APD</sup> structures

<b>Data collection</b>	Nup192 <sup>TAIL</sup>	Nup188 <sup>TAIL</sup>	Nup82 <sup>NTD</sup> Nup159 <sup>T</sup> Nup145N <sup>APD</sup>	Nup82 <sup>NTD</sup> Nup159 <sup>T</sup> Nup145N <sup>APD</sup>
Protein(s)				
PDB code	5CWV	5CWU	5CWW	
Synchrotron	SSRL <sup>a</sup>	APS <sup>b</sup>	APS	SSRL
Beamline	BL12-2	23ID-D	23ID-D	BL12-2
Space group	P2 <sub>1</sub> 2 <sub>1</sub> 2 <sub>1</sub>	P1	C2	C2
Cell parameters				
<i>a</i> (Å)	64.7	65.2	122.3	122.1
<i>b</i> (Å)	114.4	65.3	108	108.2
<i>c</i> (Å)	114.7	155.5	69.6	69.6
$\alpha$ (°)	90.0	90.0	90.0	90.0
$\beta$ (°)	90.0	89.9	108.6	108.4
$\gamma$ (°)	90.0	90.0	90.0	90.0
	<i>Se peak</i>	<i>Se peak</i>	<i>Native</i>	<i>Se peak</i>
Wavelength (Å)	0.9793	0.9794	1.0000	0.97940
Resolution (Å)	20.0 – 3.15	20.0 – 3.35	30.0 – 2.2	40.0 – 2.5
<i>R</i> <sub>sym</sub> (%) <sup>c</sup>	8.1 (100.4)	7.0 (98.6)	8.4 (154.6)	6.9 (68.4)
<i>R</i> <sub>pim</sub> (%) <sup>c</sup>	2.5 (34.6)	2.6 (38.3)	3.2 (69.4)	2.9 (28.5)
<i>CC</i> <sub>1/2</sub> (%) <sup>c</sup>	99.9 (69.2)	100.0 (80.4)	99.9 (58.4)	99.9 (86.4)
<i>&lt;I&gt; / &lt;σI&gt;</i> <sup>c</sup>	28.3 (2.2)	34.0 (2.1)	13.6 (1.2)	15.0 (2.5)
Completeness (%) <sup>c</sup>	99.5 (99.2)	99.3 (98.6)	99.0 (97.1)	98.6 (98.1)
No. observations	183,279	294,471	300,689	202,782
No. unique reflections <sup>c,d</sup>	28,157 (2,819)	74,517 (7,493)	43,372 (6,846)	57,257 (9,135)
Redundancy <sup>c</sup>	12.2 (9.2)	7.9 (7.7)	6.9 (6.7)	6.9 (7.0)
Wilson B factor	113	130	53	68
<b>Refinement</b>				
Resolution (Å)	20.0 – 3.15	20.0 – 3.35	30.0 – 2.2	
No. reflections total	28,095	72,752	43,203	
No. reflections test set	2,843 (10.1%)	3,632 (5.0%)	2,959 (6.8%)	
<i>R</i> <sub>work</sub> / <i>R</i> <sub>free</sub>	18.5 / 24.1	24.6 / 27.9	18.5 / 21.3	
No. atoms	4,167	18,312	5,785	
Protein	4,167	18,264	5,614	
Ligand	0	48	0	
Water	0	0	171	
<i>B</i> -factors				
Protein	121	104	75	
Ligand	N/A	80	N/A	
Water	N/A	N/A	61	
R.m.s. deviations				
Bond lengths (Å)	0.003	0.004	0.003	
Bond angles (°)	0.6	0.7	0.8	
Ramachandran plot <sup>e</sup>				
Favored (%)	94.2	89.4	97.1	
Additionally allowed (%)	5.8	10.6	2.9	
Outliers (%)	0.0	0.0	0.0	

<sup>a</sup> SSRL, Stanford Synchrotron Radiation Lightsource<sup>b</sup> APS, Advanced Photon Source<sup>c</sup> Highest-resolution shell is shown in parentheses<sup>d</sup> Unique reflections distinguish anomalous Friedel pairs<sup>e</sup> As determined by MolProbity (61)

Table S3.

## Crystallographic analysis of channel nup fragment structures

<b>Data collection</b>	<i>hsNup57</i> <sup>CCS3*</sup>	<i>hsNup57</i> <sup>CCS3*</sup>	<i>hsNup49</i> <sup>CCS2+3*</sup>	<i>hsNup49</i> <sup>CCS2+3*</sup> <i>hsNup57</i> <sup>CCS3*</sup>	<i>hsNup49</i> <sup>CCS2+3*</sup> <i>hsNup57</i> <sup>CCS3*</sup>	<i>Nup57</i> <sup>CCS3*</sup>	<i>Nup57</i> <sup>CCS3*</sup> I289M
Protein 1							
Protein 2							
Stoichiometry	Homotetramer	Homotetramer	Homotetramer	2:2 stoichiometry	1:2 stoichiometry	Homodimer	Homotetramer
PDB code	4JNV	4JNU	4JQ5	4JO7	4JO9	5CWT	
Synchrotron	SSRL <sup>a</sup>	ALS <sup>b</sup>	SSRL	SSRL	SSRL	SSRL	SSRL
Beamline	BL12-2	BL8.2.1	BL12-2	BL12-2	BL12-2	BL12-2	BL12-2
Space group	C2	P2 <sub>1</sub>	P1	P1	P4 <sub>1</sub> 22	P2 <sub>1</sub> 2 <sub>1</sub> 2 <sub>1</sub>	P2 <sub>1</sub> 2 <sub>1</sub> 2 <sub>1</sub>
Cell parameters							
<i>a</i> (Å)	70.5	39.9	63.8	47.7	55.5	42.3	42.2
<i>b</i> (Å)	36.6	45.1	77.1	47.7	55.5	42.4	42.2
<i>c</i> (Å)	63.2	46.4	77.2	71.3	191.2	138.7	139.6
$\alpha$ (°)	90.0	90.0	120.0	88.9	90.0	90.0	90.0
$\beta$ (°)	104.4	113.8	90.1	83.4	90.0	90.0	90.0
$\gamma$ (°)	90.0	90.0	89.9	81.4	90.0	90.0	90.0
	<i>Se peak</i>	<i>Native</i>	<i>Native</i>	<i>Se peak</i>	<i>Native</i>	<i>Native</i>	<i>Se peak</i>
Wavelength (Å)	0.9794	1.0000	1.0000	0.9794	1.0000	1.00010	0.9792
Resolution (Å)	20.0 – 1.85	20.0 – 1.45	20.0 – 2.2	50.0 – 1.75	50.0 – 2.5	20.0 – 2.5	20.0 – 2.75
<i>R</i> <sub>sym</sub> (%) <sup>c</sup>	9.6 (54.4)	9.9 (59.0)	6.4 (52.8)	14.0 (80.2)	13.6 (74.3)	4.3 (24.2)	15.1 (84.4)
<i>R</i> <sub>plim</sub> (%) <sup>c</sup>	4.0 (24.7)	4.1 (30.2)	3.9 (32.1)	8.9 (52.7)	3.5 (22.6)	1.4 (7.0)	4.7 (28.4)
<i>CC</i> <sub>1/2</sub> (%) <sup>c</sup>	99.2 (88.6)	99.1 (88.7)	99.3 (90.8)	99.0 (46.4)	99.9 (93.8)	99.9 (99.3)	98.9 (91.6)
$\langle I \rangle / \langle \sigma \rangle$ <sup>c</sup>	16.0 (4.1)	15.5 (2.2)	17.8 (2.1)	10.0 (2.2)	19.8 (2.1)	58.3 (6.3)	14.8 (2.7)
Completeness (%) <sup>c</sup>	97.3 (97.2)	97.2 (81.6)	97.3 (87.7)	97.5 (95.3)	98.8 (89.1)	98.0 (88.2)	99.7 (98.8)
No. observations	82,655	174,150	242,553	205,417	169,333	136,374	79,570
No. unique reflections <sup>c,d</sup>	25,273 (2,494)	26,320 (2,198)	63,140 (5,724)	117,121 (11,189)	11,003 (953)	8,970 (759)	12,635 (1,297)
Redundancy <sup>c</sup>	6.3 (5.3)	6.6 (3.6)	3.8 (3.5)	3.4 (3.1)	15.4 (10.0)	15.2 (10.2)	11.4 (8.3)
Wilson B factor	32	18	45	17	46	32	71
<b>Refinement</b>							
Resolution (Å)	20.0 – 1.85	20.0 – 1.45	20.0 – 2.2	50.0 – 1.75	50.0 – 2.5	20.0 – 2.5	
No. reflections total	25,266	26,274	63,070	116,939	10,891	8,126	
No. reflections test set	2,508 (9.93%)	1,990 (7.57%)	1,990 (3.16%)	3,925 (3.36%)	1,092 (10.0%)	809 (9.9%)	
<i>R</i> <sub>work</sub> / <i>R</i> <sub>free</sub>	23.7 / 27.7	18.3 / 21.8	28.5 / 29.3	18.6 / 21.4	24.7 / 29.4	24.1 / 29.3	
No. atoms	1,108	3,121	8,526	4,592	1,355	1,323	
Protein	1,086	2,989	8,526	4,138	1,338	1,305	
Ligand	0	0	0	0	0	0	
Water	22	132	0	454	17	18	
<i>B</i> -factors							
Protein	30	25	51	19	89	58	
Ligand	N/A	N/A	N/A	N/A	N/A	N/A	
Water	30	37	N/A	32	61	54	
R.m.s. deviations							
Bond lengths (Å)	0.009	0.007	0.002	0.008	0.002	0.002	
Bond angles (°)	0.9	1.0	0.5	0.8	0.4	0.4	
Ramachandran plot <sup>e</sup>							
Favored (%)	99.2	100.0	96.1	99.2	97.4	99.3	
Additionally allowed (%)	0.8	0.0	3.9	0.8	2.6	0.7	
Outliers (%)	0.0	0.0	0.0	0.0	0.0	0.0	

<sup>a</sup> SSRL, Stanford Synchrotron Radiation Lightsources<sup>b</sup> ALS, Advanced Light Source<sup>c</sup> Highest-resolution shell is shown in parentheses<sup>d</sup> Unique reflections distinguish anomalous Friedel pairs<sup>e</sup> As determined by MolProbity (61)

**Table S4.****Crystallographic analysis of the CNT•Nic96<sup>R1</sup>•sAB-158 structure**

<b>Data collection</b>	<b>K<sub>2</sub>OsCl<sub>6</sub><sup>g</sup></b>	<b>K<sub>2</sub>OsCl<sub>6</sub><sup>g</sup></b>	<b>K<sub>2</sub>OsO<sub>4</sub></b>	<b>SeMet<sup>h</sup></b>	<b>PCMB</b>	<b>EMTS</b>	<b>Native</b>
Synchrotron	SSRL <sup>a</sup>	SSRL	SSRL	SSRL	SSRL	SSRL	APS <sup>b</sup>
Beamline	BL12-2	BL12-2	BL12-2	BL12-2	BL12-2	BL12-2	23ID-D
Space group	P2 <sub>1</sub> 2 <sub>1</sub> 2 <sub>1</sub>	P2 <sub>1</sub> 2 <sub>1</sub> 2 <sub>1</sub>	P2 <sub>1</sub> 2 <sub>1</sub> 2 <sub>1</sub>	P2 <sub>1</sub> 2 <sub>1</sub> 2 <sub>1</sub>	P2 <sub>1</sub> 2 <sub>1</sub> 2 <sub>1</sub>	P2 <sub>1</sub> 2 <sub>1</sub> 2 <sub>1</sub>	P2 <sub>1</sub> 2 <sub>1</sub> 2 <sub>1</sub>
Cell dimensions							
<i>a</i> (Å)	123.2	123.2	122.5	122.9	123.3	121.5	123.7
<i>b</i> (Å)	162.8	162.8	162.8	162.2	163.8	162.4	162.9
<i>c</i> (Å)	212.9	212.9	215.2	214.0	214.5	215.5	214.1
$\alpha$ (°)	90.0	90.0	90.0	90.0	90.0	90.0	90.0
$\beta$ (°)	90.0	90.0	90.0	90.0	90.0	90.0	90.0
$\gamma$ (°)	90.0	90.0	90.0	90.0	90.0	90.0	90.0
Wavelength (Å)	<i>Os peak</i> 1.1400	<i>Os peak</i> 1.1400	<i>Os peak</i> 1.1400	<i>Se peak</i> 0.9793	<i>Hg peak</i> 1.0078	<i>Hg peak</i> 1.0054	<i>Native</i> 1.0330
Resolution (Å)	50.0 – 3.77	50.0 – 3.95	40.0 – 4.4	40.0 – 3.77	40.0 – 5.6	40.0 – 4.8	40.0 – 3.95
<i>R</i> <sub>meas</sub> (%) <sup>c</sup>	20.4 (585.7)	17.2 (308.4)	16.3 (302.1)	18.1 (495.9)	11.9 (330.3)	15.5 (304.6)	11.3 (373.9)
<i>R</i> <sub>pim</sub> (%) <sup>c</sup>	3.2 (91.9)	2.7 (53.1)	3.1 (63.7)	3.5 (99.8)	3.3 (95.8)	4.2 (89.3)	2.2 (83.2)
<i>CC</i> <sub>1/2</sub> (%) <sup>c</sup>	100.0 (54.4)	100.0 (74.6)	100.0 (57.2)	100.0 (57.6)	99.9 (53.0)	99.0 (64.7)	100.0 (59.5)
$\langle I \rangle / \langle \sigma \rangle$ <sup>c</sup>	17.0 (1.00)	19.36 (1.84)	14.26 (1.42)	14.49 (1.01)	14.31 (1.02)	11.68 (1.16)	17.79 (1.17)
Completeness (%) <sup>c</sup>	99.9 (99.9)	99.9 (100.0)	99.7 (100.0)	99.8 (99.8)	99.4 (99.8)	98.7 (97.9)	99.5 (98.9)
No. of observations	1,765,669	1,538,483	740,306	1,167,642	181,671	283,261	1,024,643
No. of unique reflections <sup>c,d</sup>	83,402 (13,626)	72,513 (11,756)	52,614 (8,566)	83,196 (13,534)	25,719 (4,067)	39,850 (6,414)	38,491 (6,087)
Redundancy <sup>c</sup>	40.2 (40.2)	40.2 (40.4)	26.5 (27.7)	26.7 (27.1)	13.1 (13.8)	13.4 (13.1)	26.6 (26.0)
Wilson B factor	178	169	235	160	413	288	204
<b>Refinement</b>							
Resolution (Å)	50 – 3.77						
No. of reflections	83,342						
No. of reflections test set	3,789 (4.56%)						
<i>R</i> <sub>work</sub> / <i>R</i> <sub>free</sub>	22.9 / 26.5						
No. atoms	16,564						
Protein	16,562						
Ions	2						
R.m.s deviations							
Bond lengths (Å)	0.003						
Bond angles (°)	0.7						
<i>B</i> -factors <sup>e</sup>							
Protein	242						
Ions	299						
Ramachandran plot <sup>f</sup>							
Favored (%)	93.1						
Additionally allowed (%)	6.9						
Outliers (%)	0.0						

<sup>a</sup> SSRL, Stanford Synchrotron Radiation Lightsource<sup>b</sup> APS, Advanced Photon Source<sup>c</sup> Highest-resolution shell is shown in parentheses<sup>d</sup> Unique reflections distinguish anomalous Friedel pairs<sup>e</sup> *B*-factors include overall *B*-factor of the crystal<sup>f</sup> As determined by MolProbity (61)<sup>g</sup> As *reference*, data processing statistics using different high resolution cutoffs are indicated.<sup>h</sup> Representative statistics for all 23 SeMet datasets (wild type and 22 methionine mutants)

**Table S5.****Bacterial expression constructs and expression conditions**

#	Protein	Residues	Expression vector	Restriction sites 5', 3'	N-terminal overhang	C-terminal overhang	Expression conditions
1*	hsNup49 CCS2+3*	341-428	pET28a-Sumo	BamHI, NotI	S	none	18 °C / 18 hours
2	hsNup49	341-428 (Y420F)	pET28a-Sumo	BamHI, NotI	S	none	18 °C / 18 hours
3*	hsNup57 CCS3*	453-491	pET28a-Sumo	BamHI, NotI	S	none	18 °C / 18 hours
4*	hsNup57	453-491 (I478M)	pET28a-Sumo	BamHI, NotI	S	none	18 °C / 18 hours
5*	Nsp1 Nup57	467-674 74-319	pETDuet1	NcoI/NotI NdeI/XhoI	None M	none none	18 °C / 18 hours
6	Nsp1 Nup57 $\Delta\alpha/\beta$	467-674 146-319	pETDuet1	NcoI/NotI NdeI/XhoI	None M	none none	18 °C / 18 hours
7**	Nsp1 Nup57	467-674 (L527M) 74-319	pETDuet1	NcoI/NotI	None M	none	18 °C / 18 hours
8**	Nsp1 Nup57	467-674 (L548M) 74-319	pETDuet1	NdeI/XhoI	None M	none	18 °C / 18 hours
9**	Nsp1 Nup57	467-674 (A628M) 74-319	pETDuet1	NcoI/NotI	None M	none	18 °C / 18 hours
10**	Nsp1 Nup57	467-674 74-319 (I82M)	pETDuet1	NdeI/XhoI	None M	none	18 °C / 18 hours
11**	Nsp1 Nup57	467-674 74-319 (H109M)	pETDuet1	NcoI/NotI	None M	none	18 °C / 18 hours
12**	Nsp1 Nup57	467-674 74-319 (L113M)	pETDuet1	NdeI/XhoI	None M	none	18 °C / 18 hours
13**	Nsp1 Nup57	467-674 74-319 (E127M)	pETDuet1	NcoI/NotI	None M	none	18 °C / 18 hours
14**	Nsp1 Nup57	467-674 74-319 (I138M)	pETDuet1	NcoI/NotI	None M	none	18 °C / 18 hours
15**	Nsp1 Nup57	467-674 74-319 (L198M)	pETDuet1	NcoI/NotI	None M	none	18 °C / 18 hours
16**	Nsp1 Nup57	467-674 74-319 (I233M)	pETDuet1	NcoI/NotI	None M	none	18 °C / 18 hours
17**	Nsp1 Nup57	467-674 74-319 (L253M)	pETDuet1	NcoI/NotI	None M	none	18 °C / 18 hours
18**	Nsp1 Nup57	467-674 74-319 (V303M)	pETDuet1	NcoI/NotI	None M	none	18 °C / 18 hours
19	Nsp1	467-678	pET28a-Sumo	BamHI, NotI	S	none	18 °C / 18 hours
20	Nup57	74-326	pGEX-6P1	BamHI, NotI	GPLGS	none	18 °C / 18 hours
21*	Nup57	265-317	pET28a-Sumo	BamHI, NotI	S	none	18 °C / 18 hours
22*	Nup57	265-317 (I289M)	pET28a-Sumo	BamHI, NotI	S	none	18 °C / 18 hours
23*	Nup49	246-470	pET28a-Sumo	BamHI, NotI	S	none	18 °C / 18 hours
24**	Nup49	246-470 (I263M)	pET28a-Sumo	BamHI, NotI	S	none	18 °C / 18 hours
25**	Nup49	246-470 (H276M)	pET28a-Sumo	BamHI, NotI	S	none	18 °C / 18 hours
26**	Nup49	246-470 (L301M)	pET28a-Sumo	BamHI, NotI	S	none	18 °C / 18 hours
27**	Nup49	246-470 (A307M)	pET28a-Sumo	BamHI, NotI	S	none	18 °C / 18 hours
28**	Nup49	246-470 (L332M)	pET28a-Sumo	BamHI, NotI	S	none	18 °C / 18 hours
29**	Nup49	246-470 (E438M)	pET28a-Sumo	BamHI, NotI	S	none	18 °C / 18 hours
30#	Avi-Nup49	246-470	pET28a-PreS	BamHI, NotI	GPLMSGSLNDIFEAKI EWHEGSAGGSGHM	none	18 °C / 18 hours
31	Nup49 E	246-470 (T378E)	pET28a-Sumo	BamHI, NotI	S	none	18 °C / 18 hours
32	Nup49 P1	246-470 (I393P)	pET28a-Sumo	BamHI, NotI	S	none	18 °C / 18 hours
33	Nup49 P2	246-470 (S442P)	pET28a-Sumo	BamHI, NotI	S	none	18 °C / 18 hours
34	Nup49 PP	246-470 (I393P, S442P)	pET28a-Sumo	BamHI, NotI	S	none	18 °C / 18 hours
35	Nup49 EPP	246-470 (T378E, I393P, S442P)	pET28a-Sumo	BamHI, NotI	S	none	18 °C / 18 hours
36	Nup49 EVPIP	246-470 (T378E, T292V, I393P, L400I, S442P)	pET28a-Sumo	BamHI, NotI	S	none	18 °C / 18 hours
37	Nic96	110-1112	pET28a-Sumo	BamHI, NotI	S	AAALEHHHHHH	18 °C / 18 hours
38#	Nic96	139-201	pET28a-Sumo	BamHI, NotI	S	none	18 °C / 18 hours
39**	Nic96 R1	139-180	pET28a-Sumo	BamHI, NotI	S	none	18 °C / 18 hours
40**	Nic96 R1	139-180 (L178M)	pET28a-Sumo	BamHI, NotI	S	none	18 °C / 18 hours
41**	Nic96 R1	139-180 (L140M)	pET28a-Sumo	BamHI, NotI	S	none	18 °C / 18 hours
42**	Nic96 R1	139-180 (L161M)	pET28a-Sumo	BamHI, NotI	S	none	18 °C / 18 hours
43**	Nic96 R1	139-180 (L164M)	pET28a-Sumo	BamHI, NotI	S	none	18 °C / 18 hours
44	Nic96	139-164	pET28a-Sumo	BamHI, NotI	S	none	18 °C / 18 hours
45	Nic96	139-171	pET28a-Sumo	BamHI, NotI	S	none	18 °C / 18 hours
46	Nic96	146-180	pET28a-Sumo	BamHI, NotI	S	none	18 °C / 18 hours
47	Nic96	156-180	pET28a-Sumo	BamHI, NotI	S	none	18 °C / 18 hours
48	Nic96	165-180	pET28a-Sumo	BamHI, NotI	S	none	18 °C / 18 hours
49	Nic96	139-180 (L168A, L171A, L175A, L178A)	pET28a-Sumo	BamHI, NotI	S	none	18 °C / 18 hours
50	Nic96	139-180 (L168A, L171A)	pET28a-Sumo	BamHI, NotI	S	none	18 °C / 18 hours
51	Nic96	139-180 (L175A, L178A)	pET28a-Sumo	BamHI, NotI	S	none	18 °C / 18 hours
52	Nic96 R1-R2	139-301	pET-MCN-Sumo	BamHI, NotI	S	none	N/A
53	Nic96 R2-SOL	245-1112	pGEX-6P1	BamHI, NotI	GPHM	none	23 °C / 18 hours

#	Protein	Residues	Expression vector	Restriction sites 5', 3'	N-terminal overhang	C-terminal overhang	Expression conditions
54	Nic96 SOL	391-1112	pET28a-SUMO	BamHI, NotI	S	none	18 °C / 18 hours
55	Nic96 R2	245-301	pET-MCN-Sumo	BamHI, NotI	S	none	37 °C / 2 hours
56	Nic96 R2	245-301 (F275A)	pET-MCN-Sumo	BamHI, NotI	S	none	37 °C / 2 hours
57	Nic96 R2	245-301 (D276A)	pET-MCN-Sumo	BamHI, NotI	S	none	37 °C / 2 hours
58	Nic96 R2	245-301 (F278A)	pET-MCN-Sumo	BamHI, NotI	S	none	37 °C / 2 hours
59	Nic96 R2	245-301 (N282A)	pET-MCN-Sumo	BamHI, NotI	S	none	37 °C / 2 hours
60	Nic96 R2	245-301 (L285A)	pET-MCN-Sumo	BamHI, NotI	S	none	37 °C / 2 hours
61	Nic96 R2	245-301 (W287A)	pET-MCN-Sumo	BamHI, NotI	S	none	37 °C / 2 hours
62	Nic96 R2	245-301 (I294A)	pET-MCN-Sumo	BamHI, NotI	S	none	37 °C / 2 hours
63	Nic96 R2	245-301 (F298A)	pET-MCN-Sumo	BamHI, NotI	S	none	37 °C / 2 hours
64	MB1	1-91	pET28a-Sumo	BamHI, NotI	S	none	18 °C / 18 hours
65	MB2	1-93	pET28a-Sumo	BamHI, NotI	S	none	18 °C / 18 hours
66	MB3	1-91	pET28a-Sumo	BamHI, NotI	S	none	18 °C / 18 hours
67	MB4	1-93	pET28a-Sumo	BamHI, NotI	S	none	18 °C / 18 hours
68	MB5	1-93	pET28a-Sumo	BamHI, NotI	S	none	18 °C / 18 hours
69	MB6	1-92	pET28a-Sumo	BamHI, NotI	S	none	18 °C / 18 hours
70	MB7	1-91	pET28a-Sumo	BamHI, NotI	S	none	18 °C / 18 hours
71	MB8	1-93	pET28a-Sumo	BamHI, NotI	S	none	18 °C / 18 hours
72	MB9	1-91	pET28a-Sumo	BamHI, NotI	S	none	18 °C / 18 hours
73	MB10	1-93	pET28a-Sumo	BamHI, NotI	S	none	18 °C / 18 hours
74*	sAB-158-LC sAB-158-HC	1-204 1-258	pSV4	NcoI, Sall	none	VDKKVEPKSCDKT HTGGSHHHHHH	37 °C / 2 hours
75	sAB-160-LC sAB-160-HC	1-204 1-258	pSV4	NcoI, Sall	none	VDKKVEPKSCDKT HTGGSHHHHHH	37 °C / 2 hours
76	Nup145N	606-993	pET28a-Sumo	BamHI, NotI	S	none	23 °C / 18 hours
77*	Nup145N APD	858-993	pET28a-PreS	NdeI, XhoI	GPHM	none	23 °C / 18 hours
78	Nup53 ΔC	1-361	pET28a-Sumo	BamHI, XhoI	S	LEHHHHHH	23 °C / 18 hours
79*	Nup82 NTD	1-595	pET28a-PreS	NdeI, NotI	GPH	none	18 °C / 18 hours
80*	Nup159 T	1440-1481	pET28a-Sumo	BamHI, NotI	S	none	18 °C / 18 hours
81	Nup192	1-1756	pET28a-PreS	NdeI, NotI	GPLMSGSLNDIFEAKI EWHEGSAGGSGH	none	23 °C / 18 hours
82	Nup192 CTD	944-1756	pET28a-PreS	NdeI, NotI	GPHM	none	23 °C / 18 hours
83*	Nup192 TAIL	1397-1756	pET28a-PreS	NdeI, NotI	GPHM	none	37 °C / 2 hours
84*	Nup192 TAIL	1397-1756 (L1403E, I1427E)	pET28a-PreS	NdeI, NotI	GPHM	none	37 °C / 2 hours
85	Nup192 TAIL	1397-1756 (L1597A)	pET28a-PreS	NdeI, NotI	GPHM	none	37 °C / 2 hours
86	Nup192 TAIL	1397-1756 (F1602A)	pET28a-PreS	NdeI, NotI	GPHM	none	37 °C / 2 hours
87	Nup192 TAIL	1397-1756 (F1602W)	pET28a-PreS	NdeI, NotI	GPHM	none	37 °C / 2 hours
88	Nup192 TAIL	1397-1756 (F1611A)	pET28a-PreS	NdeI, NotI	GPHM	none	37 °C / 2 hours
89	Nup192 TAIL	1397-1756 (D1614A, P1615A, E1616A)	pET28a-PreS	NdeI, NotI	GPHM	none	37 °C / 2 hours
90	Nup192 TAIL	1397-1756 (D1614A, E1616A)	pET28a-PreS	NdeI, NotI	GPHM	none	37 °C / 2 hours
91	Nup192 TAIL	1397-1756 (F1664A)	pET28a-PreS	NdeI, NotI	GPHM	none	37 °C / 2 hours
92	Nup192 TAIL	1397-1756 (F1664W)	pET28a-PreS	NdeI, NotI	GPHM	none	37 °C / 2 hours
93	Nup192 TAIL	1397-1756 (V1673A)	pET28a-PreS	NdeI, NotI	GPHM	none	37 °C / 2 hours
94	Nup192 TAIL	1397-1756 (L1676A)	pET28a-PreS	NdeI, NotI	GPHM	none	37 °C / 2 hours
95	Nup192 TAIL	1397-1756 (K1677A)	pET28a-PreS	NdeI, NotI	GPHM	none	37 °C / 2 hours
96	Nup192 TAIL	1397-1756 (M1727A)	pET28a-PreS	NdeI, NotI	GPHM	none	37 °C / 2 hours
97	Nup192 TAIL	1397-1756 (I1730A)	pET28a-PreS	NdeI, NotI	GPHM	none	37 °C / 2 hours
98	Nup192 TAIL	1397-1756 (F1735A)	pET28a-PreS	NdeI, NotI	GPHM	none	37 °C / 2 hours
99	Nup192 TAIL	1397-1756 (Y1738A)	pET28a-PreS	NdeI, NotI	GPHM	none	37 °C / 2 hours
100*	Nup188 TAIL	1447-1858	pET28a-SUMO	BamHI, NotI	SM	none	18 °C / 18 hours

\* Nup192 TAIL used for SEC-MALS analyses harbored two mutations to prevent non-physiological dimerization. These mutants were designed based on observed crystal packing interactions in the Nup192<sup>TAIL</sup> structure

\* Constructs that were used for crystallization

# Constructs that were used for sAB and MB selection

▪ Constructs that were used for ascertaining sequence register by Se-Met labeling



**Table S6.****Protein purification protocols**

Protein(s)	Expression constructs	Purification step	Buffer A	Buffer B
Nup192 TAIL*	Individual (#83)	1. Ni-NTA 2. Dialysis/Cleavage 3. Ni-NTA 4. HiLoad Superdex 200 16/60 PG	Ni-A1, 5 mM $\beta$ -ME Ni-A3, 5 mM $\beta$ -ME / PreS Ni-A3, 5 mM $\beta$ -ME SEC-B, 1 mM DTT	Ni-B1, 5 mM $\beta$ -ME N/A Ni-B1, 5 mM $\beta$ -ME N/A
His <sub>6</sub> -Nup192 TAIL wild type and mutants	Individual (#83, #85-99)	1. Ni-NTA 2. Dialysis 3. HiTrap Q HP 4. HiLoad Superdex 200 16/60 PG	Ni-A1, 5 mM $\beta$ -ME IEX-A1, pH 9.0, 5 mM $\beta$ -ME IEX-A1, pH 9.0, 1 mM DTT SEC-B, 1 mM DTT	Ni-B1, 5 mM $\beta$ -ME N/A IEX-B, pH 9.0, 1 mM DTT N/A
His <sub>6</sub> -Nup192 TAIL•SUMO-Nic96 R1-R2	Co-expression (#84+#52)	1. Ni-NTA 2. Dialysis 3. HiTrap Q HP 4. HiLoad Superdex 200 16/60 PG	Ni-A1, 5 mM $\beta$ -ME IEX-A1, pH 9.0, 5 mM $\beta$ -ME IEX-A1, pH 9.0, 1 mM DTT SEC-B, 1 mM DTT	Ni-B1, 5 mM $\beta$ -ME N/A IEX-B, pH 9.0, 1 mM DTT N/A
Nup188 TAIL*	Individual (#100)	1. Ni-NTA 2. Dialysis/Cleavage 3. Ni-NTA 4. MonoQ 10/100 GL 5. HiLoad Superdex 200 16/60 PG	Ni-A1, 5 mM $\beta$ -ME Ni-A2, 5 mM $\beta$ -ME / ULP1 Ni-A2, 5 mM $\beta$ -ME IEX-A2, pH 8.0, 1 mM DTT SEC-A, 1 mM DTT	Ni-B1, 5 mM $\beta$ -ME N/A Ni-B1, 5 mM $\beta$ -ME IEX-B, pH 8.0, 1 mM DTT N/A
SUMO-Nup188 TAIL	Individual (#100)	1. Ni-NTA 2. Dialysis 3. MonoQ 10/100 GL 4. HiLoad Superdex 200 16/60 PG	Ni-A1, 5 mM $\beta$ -ME IEX-A2, pH 8.0, 5 mM $\beta$ -ME IEX-A2, pH 8.0, 1 mM DTT SEC-B, 1 mM DTT	Ni-B1, 5 mM $\beta$ -ME N/A IEX-B, pH 8.0, 1 mM DTT N/A
SUMO-Nup188 TAIL•SUMO-Nic96 R1-R2	Co-expression (#100+#52)	1. Ni-NTA 2. Dialysis 3. MonoQ 10/100 GL 4. HiLoad Superdex 200 16/60 PG	Ni-A1, 5 mM $\beta$ -ME IEX-A2, pH 8.0, 5 mM $\beta$ -ME IEX-A2, pH 8.0, 1 mM DTT SEC-B, 1 mM DTT	Ni-B1, 5 mM $\beta$ -ME N/A IEX-B, pH 8.0, 1 mM DTT N/A
SUMO-Nic96 R1* wild type, mutants, and truncations	Individual (#39-#51)	1. Ni-NTA 2. HiPrep 26/20 Desalting 3. HiLoad Superdex 75 16/60 PG	Ni-A1, 5 mM $\beta$ -ME SEC-A, 5 mM DTT SEC-A, 5 mM DTT	Ni-B1, 5 mM $\beta$ -ME N/A N/A
SUMO-Nic96 R2 wild type and mutants	Individual (#55-63)	1. Ni-NTA 2. Dialysis 3. HiTrap Q HP 4. HiLoad Superdex 75 16/60 PG	Ni-A1, 5 mM $\beta$ -ME IEX-A2, pH 8.0, 5 mM $\beta$ -ME IEX-A2, pH 8.0, 1 mM DTT SEC-B, 1 mM DTT	Ni-B1, 5 mM $\beta$ -ME N/A IEX-B, pH 8.0, 1 mM DTT N/A
Nup145N	Individual (#76)	1. Ni-NTA 2. Dialysis/Cleavage 3. MonoS 5/50 GL 4. HiLoad Superdex 75 16/60 PG	Ni-A1, 5 mM $\beta$ -ME IEX-A2, pH 7.0, 5 mM DTT / ULP1 IEX-A2, pH 7.0, 5 mM DTT SEC-A, 5 mM DTT	Ni-B1, 5 mM $\beta$ -ME N/A IEX-B, pH 7.0, 5 mM DTT N/A
Nup145N APD*	Individual (#77)	1. Ni-NTA 2. Dialysis/cleavage 3. Ni-NTA 4. Superdex 75 16/60 PG	Ni-A1, 5 mM $\beta$ -ME IEX-A2, pH 8.0, 5 mM $\beta$ -ME / PreS Ni-A2, 5 mM $\beta$ -ME SEC-A, 5 mM DTT	Ni-B1, 5 mM $\beta$ -ME N/A Ni-B1, 5 mM $\beta$ -ME N/A
Nup53	Individual (#78)	1. Ni-NTA 2. HiPrep 26/20 Desalting/Cleavage 3. Ni-NTA 4. HiPrep 26/20 Desalting 5. MonoS 5/50 GL 6. HiLoad Superdex 75 16/60 PG	Ni-A1, 5 mM $\beta$ -ME Ni-A1, 5 mM $\beta$ -ME / ULP1 Ni-A1, 5 mM $\beta$ -ME IEX-A2, pH 7.0, 5 mM DTT IEX-A2, pH 7.0, 5 mM DTT SEC-A, 5 mM DTT	Ni-B1, 5 mM $\beta$ -ME N/A Ni-B1, 5 mM $\beta$ -ME N/A IEX-B, pH 7.0, 5 mM DTT N/A
hsNup49 CCS2+3** wild type and mutant	Individual (#1-2)	1. Ni-NTA 2. HiPrep 26/20 Desalting/Cleavage 3. Ni-NTA 4. HiLoad Superdex 75 16/60 PG	Ni-A1, 5 mM $\beta$ -ME SEC-A, 4 mM $\beta$ -ME / ULP1 Ni-A2, pH 8.0, 4 mM $\beta$ -ME SEC-A, 4 mM DTT	Ni-B1, 5 mM $\beta$ -ME N/A Ni-B1, 5 mM $\beta$ -ME N/A
hsNup57 CCS3** wild type and mutant	Individual (#3-4)	1. Ni-NTA 2. HiPrep 26/20 Desalting/Cleavage 3. Ni-NTA 4. HiLoad Superdex 75 16/60 PG	Ni-A1, 5 mM $\beta$ -ME SEC-A, 4 mM $\beta$ -ME / ULP1 Ni-A2, pH 8.0, 4 mM $\beta$ -ME SEC-A, 4 mM DTT	Ni-B1, 5 mM $\beta$ -ME N/A Ni-B1, 5 mM $\beta$ -ME N/A
ctNup57 CCS3** wild type and mutant	Individual (#21-22)	1. Ni-NTA 2. HiPrep 26/20 Desalting/Cleavage 3. Ni-NTA 4. HiLoad Superdex 75 16/60 PG	Ni-A1, 5 mM $\beta$ -ME SEC-A, 4 mM $\beta$ -ME / ULP1 Ni-A2, pH 8.0, 4 mM $\beta$ -ME SEC-A, 4 mM DTT	Ni-B1, 5 mM $\beta$ -ME N/A Ni-B1, 5 mM $\beta$ -ME N/A
Nic96 SOL	Individual (#54)	1. Ni-NTA 2. Dialysis/Cleavage 3. Ni-NTA 4. MonoQ 10/100 GL 5. HiLoad Superdex 200 16/60 PG	Ni-A1, 5 mM $\beta$ -ME IEX-A2, pH 8.0, 5 mM $\beta$ -ME / ULP1 Ni-A2, 5 mM $\beta$ -ME IEX-A2, pH 8.0, 5 mM DTT SEC-A, 5 mM DTT	Ni-B1, 5 mM $\beta$ -ME N/A Ni-B1, 5 mM $\beta$ -ME IEX-B, pH 8.0, 5 mM DTT N/A
avi-Nup192	Individual (#81)	1. Ni-NTA 2. Dialysis/Cleavage 3. Ni-NTA 4. MonoQ 10/100 GL 5. HiLoad Superdex 200 16/60 PG	Ni-A1, 5 mM $\beta$ -ME IEX-A2, pH 8.0, 5 mM $\beta$ -ME / PreS Ni-A2, pH 8.0, 5 mM $\beta$ -ME IEX-A2, pH 8.0, 5 mM DTT SEC-A, 5 mM DTT	Ni-B1, 5 mM $\beta$ -ME N/A Ni-B1, 5 mM $\beta$ -ME IEX-B, pH 8.0, 5 mM DTT N/A
CNT* wild type mutants avi-tagged variant Met mutants	Co-expression (#5+#23) (#5+#31-36) (#5+#12) (#5+#24-29)	1. Ni-NTA 2. HiPrep 26/20 Desalting/Cleavage 3. Ni-NTA 4. HiTrap Q HP 5. HiLoad Superdex 200 16/60 PG	Ni-A1, 4 mM $\beta$ -ME IEX-A2, pH 8.0, 4 mM $\beta$ -ME / ULP1 Ni-A2, pH 8.0, 4 mM $\beta$ -ME IEX-A2, pH 8.0, 5 mM DTT SEC-A, 5 mM DTT	Ni-B1, 4 mM $\beta$ -ME N/A Ni-B1, 4 mM $\beta$ -ME IEX-B, pH 8.0, 5 mM DTT N/A
Nsp1	Individual (#19)	1. Ni-NTA 2. HiPrep 26/20 Desalting/Cleavage 3. Ni-NTA 4. HiLoad Superdex 200 16/60 PG	Ni-A1, 5 mM $\beta$ -ME SEC-A, 5 mM $\beta$ -ME / ULP1 Ni-A2, 5 mM $\beta$ -ME SEC-A, 5 mM DTT	Ni-B1, 5 mM $\beta$ -ME N/A Ni-B1, 5 mM $\beta$ -ME N/A

Protein(s)	Expression constructs	Purification step	Buffer A	Buffer B
Nup49•Nup57	Co-expression (#23+20)	1. GST-Sepharose fastflow 2. HiPrep 26/20 Desalting/Cleavage 3. Ni-NTA 4. HiPrep 26/20 Desalting/Cleavage 5. Ni-NTA 6. HiTrap Desalting	GST-A, 5 mM $\beta$ -ME IEX-A2: pH 8.0, 5 mM $\beta$ -ME / PreS Ni-A2, 5 mM $\beta$ -ME IEX-A2: pH 8.0, 5 mM $\beta$ -ME / ULP1 Ni-A2, 5 mM $\beta$ -ME SEC-A, 5 mM $\beta$ -ME	GST-B, 5 mM $\beta$ -ME N/A Ni-B2, 5 mM $\beta$ -ME N/A Ni-B2, 5 mM $\beta$ -ME N/A
Nup82 NTD•Nup159 T*	Individual (#79) (#80) co-lysis (1:1)	1. Ni-NTA 2. HiPrep 26/20 Desalting/Cleavage 3. Ni-NTA 4. HiLoad Superdex 200 16/60 PG	Ni-A1, 5 mM $\beta$ -ME Ni-A1, 5 mM $\beta$ -ME / PreS / ULP1 Ni-A1, 5 mM $\beta$ -ME SEC-A, 5 mM DTT	Ni-B1, 4 mM $\beta$ -ME N/A Ni-B1, 4 mM $\beta$ -ME N/A
SUMO-MBs	Individual (#64-73)	1. Ni-NTA 2. HiPrep 26/20 Desalting	Ni-A1, 4 mM $\beta$ -ME SEC-B, 4 mM $\beta$ -ME	Ni-B1, 4 mM $\beta$ -ME N/A
sABs	(#74-75)	Detailed description in SI methods		
CNT•Nic96 CNT Nic96	Individual (#5+23) (#37) co-lysis (1:1)	Detailed description in SI methods		
Nic96 R2-SOL•avi-Nup192 Nic96 R2-SOL avi-Nup192	Individual (#53) (#81) co-lysis (1:1)	Detailed description in SI methods		
CNT•SUMO-Nic96 R1	Biochemical reconstitution	Detailed description in SI methods		
CNT•Nic96 R1•sAB-158	Biochemical reconstitution	Detailed description in SI methods		
Nup82 NTD•Nup159 T•Nup145N APD	Biochemical reconstitution	Detailed description in SI methods		

\* Constructs that were used for crystallization

Ni-A1: 20 mM TRIS (pH 8.0), 500 mM NaCl, 20 mM imidazole  
Ni-A2: 20 mM TRIS (pH 8.0), 100 mM NaCl, 20 mM imidazole  
Ni-A3: 20 mM TRIS (pH 8.0), 200 mM NaCl, 20 mM imidazole  
Ni-B1: 20 mM TRIS (pH 8.0), 500 mM NaCl, 500 mM imidazole  
Ni-B2: 20 mM TRIS (pH 8.0), 100 mM NaCl, 500 mM imidazole  
GST-A: 20 mM TRIS (pH 8.0), 300 mM NaCl  
GST-B: 20 mM TRIS (pH 8.0), 300 mM NaCl, 20 mM glutathione  
IEX-A1: 20 mM TRIS, 50 mM NaCl  
IEX-A2: 20 mM TRIS, 100 mM NaCl  
IEX-A3: 20 mM TRIS, 80 mM NaCl  
IEX-B: 20 mM TRIS, 2.0 M NaCl  
SEC-A: 20 mM TRIS (pH 8.0), 100 mM NaCl  
SEC-B: 20 mM TRIS (pH 8.0), 200 mM NaCl

**Table S7.****Yeast expression constructs**

Plasmid	Protein	Residues (Mutations)	Vector	Restriction sites 5', 3'	Selection Marker	Reference
pRS415-P <sub>Nop1</sub> -eGFP-scNUP192	scNup192	1-1683	pRS415	NotI, SacII	LEU2	(8)
pRS415-P <sub>Nop1</sub> -eGFP-scNup192 $\Delta$ TAIL	scNup192	1-1316	pRS415	NotI, SacII	LEU2	(8)
pRS415-P <sub>Nop1</sub> -eGFP-scNup192 Y1679A	scNup192	1-1683, Y1679A	pRS415	NotI, SacII	LEU2	this study
pRS415-P <sub>Nop1</sub> -mCherry-scNUP192	scNup192	1-1683	pRS415	NotI, SacII	LEU2	this study
pRS415-P <sub>Nop1</sub> -mCherry-scNup192 $\Delta$ TAIL	scNup192	1-1316	pRS415	NotI, SacII	LEU2	this study
pRS415-P <sub>Nop1</sub> -mCherry-scNup192 Y1679A	scNup192	1-1683, Y1679A	pRS415	NotI, SacII	LEU2	this study
pRS416-P <sub>Nop1</sub> -mCherry-scNUP192	scNup192	1-1683	pRS416	NotI, SacII	URA3	(8)
pRS415-P <sub>Nop1</sub> -eGFP-scNIC96	scNic96	1-839	pRS415	BamHI, NotI	LEU2	this study
pRS415-P <sub>Nop1</sub> -eGFP-scnic96 $\Delta$ SOL	scNic96	1-203	pRS415	BamHI, NotI	LEU2	this study
pRS415-P <sub>Nop1</sub> -eGFP-scnic96 R2-SOL	scNic96	107-839	pRS415	BamHI, NotI	LEU2	this study
pRS415-P <sub>Nop1</sub> -eGFP-scnic96 SOL	scNic96	204-839	pRS415	BamHI, NotI	LEU2	this study
pRS415-P <sub>Nop1</sub> -eGFP-scnic96 $\Delta$ R2	scNic96	1-108, 166-839	pRS415	BamHI, NotI	LEU2	this study
pRS415-P <sub>Nop1</sub> -eGFP-scnic96 F159A	scNic96	1-839, F159A	pRS415	BamHI, NotI	LEU2	this study
pRS415-P <sub>Nop1</sub> -eGFP-scnic96 $\Delta$ LINKER	scNic96	1-60, 107-839	pRS415	BamHI, NotI	LEU2	this study
pRS415-P <sub>Nop1</sub> -eGFP-scnic96 LLLL	scNic96	1-839, I46A, L49A, V53A, L56A	pRS415	BamHI, NotI	LEU2	this study
pRS415-P <sub>Nop1</sub> -mCherry-scNIC96	scNic96	1-839	pRS415	BamHI, NotI	LEU2	this study
pRS415-P <sub>Nop1</sub> -mCherry-scnic96 R2-SOL	scNic96	107-839	pRS415	BamHI, NotI	LEU2	this study
pRS415-P <sub>Nop1</sub> -mCherry-scnic96 R2	scNic96	1-108, 166-839	pRS415	BamHI, NotI	LEU2	this study
pRS415-P <sub>Nop1</sub> -mCherry-scnic96 F159A	scNic96	1-839, F159A	pRS415	BamHI, NotI	LEU2	this study
pRS415-P <sub>Nop1</sub> -mCherry-scnic96 $\Delta$ LINKER	scNic96	1-60, 107-839	pRS415	BamHI, NotI	LEU2	this study
pRS415-P <sub>Nop1</sub> -mCherry-scnic96 LLLL	scNic96	1-839, I46A, L49A, V53A, L56A	pRS415	BamHI, NotI	LEU2	this study
pRS416-P <sub>Nop1</sub> -mCherry-scNIC96	scNic96	1-839	pRS416	BamHI, NotI	URA3	this study
pRS415-P <sub>Nop1</sub> -eGFP-scNUP49	scNup49	1-472	pRS415	BamHI, NotI	LEU2	this study
pRS415-P <sub>Nop1</sub> -eGFP-scNup49 E	scNup49	1-472, K376E	pRS415	BamHI, NotI	LEU2	this study
pRS415-P <sub>Nop1</sub> -eGFP-scNup49 P1	scNup49	1-472, I391P	pRS415	BamHI, NotI	LEU2	this study
pRS415-P <sub>Nop1</sub> -eGFP-scNup49 P2	scNup49	1-472, L449P	pRS415	BamHI, NotI	LEU2	this study
pRS415-P <sub>Nop1</sub> -eGFP-scNup49 PP	scNup49	1-472, I391P, L449P	pRS415	BamHI, NotI	LEU2	this study
pRS415-P <sub>Nop1</sub> -eGFP-scNup49 EPP	scNup49	1-472, K376E, I391P, L449P	pRS415	BamHI, NotI	LEU2	this study
pRS415-P <sub>Nop1</sub> -eGFP-scNup49 EVPIP	scNup49	1-472, K376E, I390V, I391P, V398I, L449P	pRS415	BamHI, NotI	LEU2	this study*
pRS415-P <sub>Nop1</sub> -mCherry-scNUP49	scNup49	1-472	pRS415	BamHI, NotI	LEU2	this study
pRS415-P <sub>Nop1</sub> -mCherry-scNup49 E	scNup49	1-472, K376E	pRS415	BamHI, NotI	LEU2	this study
pRS415-P <sub>Nop1</sub> -mCherry-scNup49 P1	scNup49	1-472, I391P	pRS415	BamHI, NotI	LEU2	this study
pRS415-P <sub>Nop1</sub> -mCherry-scNup49 P2	scNup49	1-472, L449P	pRS415	BamHI, NotI	LEU2	this study
pRS415-P <sub>Nop1</sub> -mCherry-scNup49 PP	scNup49	1-472, I391P, L449P	pRS415	BamHI, NotI	LEU2	this study
RS415-P <sub>Nop1</sub> -mCherry-scNup49 EPP	scNup49	1-472, K376E, I391P, L449P	pRS415	BamHI, NotI	LEU2	this study
pRS415-P <sub>Nop1</sub> -mCherry-scNup49 EVPIP	scNup49	1-472, K376E, I390V, I391P, V398I, L449P	pRS415	BamHI, NotI	LEU2	this study*
pRS411-P <sub>RPL25</sub> -scRPL25-mCherry	scRpl25	1-142	pRS411	SacII, BamHI	MET15	this study
pRS413-P <sub>RPL25</sub> -scRPL25-mCherry	scRpl25	1-142	pRS413	SacII, BamHI	HIS3	this study
pFA6a-natNT2	N/A	N/A	pFA6a	N/A	N/A	(51)
pRS413	N/A	N/A	pRS413	N/A	HIS3	(52)

\*mutations based on nup49-313 (37)

**Table S8.****Yeast strains**

<b>Strain</b>	<b>Parental strain</b>	<b>Genotype</b>	<b>Plasmid</b>	<b>Transformed with</b>	<b>Analyses</b>	<b>Reference</b>
scnup192Δ	BY4741	MATa, <i>his3Δ1</i> , <i>leu2Δ0</i> , <i>met15Δ0</i> , <i>ura3Δ0</i> , <i>scnup192::HIS3</i>	pRS416-P <sub>Nop1</sub> <sup>-</sup> -mcherry- <i>scNUP192</i>	pRS415-P <sub>Nop1</sub> <sup>-</sup> -eGFP- <i>scNUP192</i> variants	growth analysis poly(A) <sup>+</sup> RNA export	this study
scnup192Δ	BY4741	MATa, <i>his3Δ1</i> , <i>leu2Δ0</i> , <i>met15Δ0</i> , <i>ura3Δ0</i> , <i>scnup192::HIS3</i>	pRS416-P <sub>Nop1</sub> <sup>-</sup> -mcherry- <i>scNUP192</i>	pRS415-P <sub>Nop1</sub> <sup>-</sup> -eGFP- <i>scNUP192</i> variants pRS411-P <sub>Nop1</sub> <sup>-</sup> - <i>scRPL25</i> -mcherry	scRpl25 localization	this study
scnup192Δ scNup57-GFP	BY4741 scNup57-GFP <sup>a</sup>	MATa, <i>his3Δ1</i> , <i>leu2Δ0</i> , <i>met15Δ0</i> , <i>ura3Δ0</i> , <i>scnup192::natNT2</i> , <i>scNUP57-GFP(S65T)::HIS3MX6</i>	pRS416-P <sub>Nop1</sub> <sup>-</sup> -mcherry- <i>scNUP192</i>	pRS415-P <sub>Nop1</sub> <sup>-</sup> -mcherry- <i>scNUP192</i> variants	colocalization of scNup192 variants and scNup57	this study
scnic96Δ	BY4741	MATa, <i>his3Δ1</i> , <i>leu2Δ0</i> , <i>met15Δ0</i> , <i>ura3Δ0</i> , <i>scnic96::HIS3</i>	pRS416-P <sub>Nop1</sub> <sup>-</sup> -mcherry- <i>scNIC96</i>	pRS415-P <sub>Nop1</sub> <sup>-</sup> -eGFP- <i>scNIC96</i> variants	growth analysis	this study
scnic96Δ scNup57-GFP	BY4741 scNup57-GFP <sup>a</sup>	MATa, <i>his3Δ1</i> , <i>leu2Δ0</i> , <i>met15Δ0</i> , <i>ura3Δ0</i> , <i>scnic96::natNT2</i> , <i>scNUP57-GFP(S65T)::HIS3MX6</i>	pRS416-P <sub>Nop1</sub> <sup>-</sup> -mcherry- <i>scNIC96</i>	pRS415-P <sub>Nop1</sub> <sup>-</sup> -mcherry- <i>scNIC96</i> variants	colocalization of scNic96 variants and scNup57 poly(A) <sup>+</sup> RNA export	this study
scnup49Δ	BY4741	MATa, <i>his3Δ1</i> , <i>leu2Δ0</i> , <i>met15Δ0</i> , <i>ura3Δ0</i> , <i>scnup49::natNT2</i>	pRS416-P <sub>Nop1</sub> <sup>-</sup> -mcherry- <i>scNUP49</i>	pRS415-P <sub>Nop1</sub> <sup>-</sup> -eGFP- <i>scNUP49</i> variants	growth analysis poly(A) <sup>+</sup> RNA assay	this study
scnup49Δ	BY4741	MATa, <i>his3Δ1</i> , <i>leu2Δ0</i> , <i>met15Δ0</i> , <i>ura3Δ0</i> , <i>scnup49::natNT2</i>	pRS416-P <sub>Nop1</sub> <sup>-</sup> -mcherry- <i>scNUP49</i>	pRS415-P <sub>Nop1</sub> <sup>-</sup> -eGFP- <i>scNUP49</i> variants pRS413-P <sub>Nop1</sub> <sup>-</sup> - <i>scRPL25</i> -mcherry	scRpl25 localization	this study
scnup49Δ scNup57-GFP	BY4741 scNup57-GFP <sup>a</sup>	MATa, <i>his3Δ1</i> , <i>leu2Δ0</i> , <i>met15Δ0</i> , <i>ura3Δ0</i> , <i>scnup49::natNT2</i> , <i>scNUP57-GFP(S65T)::HIS3MX6</i>	pRS416-P <sub>Nop1</sub> <sup>-</sup> -mcherry- <i>scNUP49</i>	pRS415-P <sub>Nop1</sub> <sup>-</sup> -eGFP- <i>scNUP49</i> variants	colocalization of scNup192 variants and scNup57	this study

<sup>a</sup> purchased from Invitrogen

**Table S9.**

Crystallization and cryo protection conditions

Protein(s)	Concentration	Crystallization condition	Cryo protection condition
Nup192 <sup>TAIL</sup>	10 mg/ml	50 mM HEPES, pH 7.1 15 % (w/v) PEG 200 340 mM sodium chloride (optimized by microseeding)	paratone oil
Nup188 <sup>TAIL</sup>	10 mg/ml	100 mM HEPES, pH 6.9 16 % (w/v) PEG 3,350 200 mM ammonium citrate	35 % (w/v) PEG 3,350
<i>hsNup49</i> <sup>CCS2+3*</sup> • <i>hsNup57</i> <sup>CCS3*</sup> 2:2 stoichiometry	15 mg/ml	100 mM sodium citrate, pH 5.4 0.5 M sodium chloride	25 % (v/v) ethylene glycol (stepwise, 20 %, 25 %)
<i>hsNup49</i> <sup>CCS2+3*</sup> • <i>hsNup57</i> <sup>CCS3*</sup> 1:2 stoichiometry	20 mg/ml	100 mM sodium acetate, pH 3.8 70 mM calcium chloride	20 % (v/v) glycerol
<i>hsNup49</i> <sup>CCS2+3*</sup>	8 mg/ml	28 % (v/v) dioxane 10 % (v/v) glycerol	20 % (v/v) glycerol
<i>hsNup57</i> <sup>CCS3*</sup>	16 mg/ml	1.0 M sodium acetate pH 5.3 1.9 M sodium formate	paraffin oil
Nup57 <sup>CCS3*</sup> homo-dimer	16 mg/ml	5 % (w/v) PEG 1,000 12 % (w/v) PEG 8,000	paraffin oil
Nup82 <sup>NTD</sup> •Nup159 <sup>T</sup> •Nup145N <sup>APD</sup>	30 mg/ml	100 mM lithium citrate 20 % (w/v) PEG 3,350 3 % (v/v) MPD	20 % (v/v) MPD
CNT•Nic96 <sup>R1</sup> •sAB-158	25 mg/ml	0.1M TRIS, pH 8.7 5.7 % (w/v) PEG 20,000 1 % (v/v) 1-propanol	25 % (v/v) ethylene glycol (stepwise, 2 % steps)

**Movie S1.**

**Rotating *C. thermophilum* CNT•Nic96<sup>R1</sup> monomer in cartoon representation.** CNT•Nic96<sup>R1</sup> is colored according to [Figure 4](#). sAb-158 is removed for clarity.

**Movie S2.**

**Rotating *C. thermophilum* CNT•Nic96<sup>R1</sup> monomer in surface representation.** CNT•Nic96<sup>R1</sup> is colored according to [Figure 4](#). sAb-158 is removed for clarity.

**Movie S3.**

**Rotating Nic96<sup>R1</sup> interaction with the three coiled-coil domains of the CNT.** The structure is colored according to [Figure 4](#). Nic96<sup>R1</sup> is shown in surface representation and Nsp1, Nup49, and Nup57 are shown in ribbon representation. The Nup49 residues that are mutated in the EPP mutant are shown in ball-and-stick representations and colored in magenta.

**Movie S4.**

**Rotating structure of the *C. thermophilum* CNT•Nic96<sup>R1</sup> asymmetric unit dimer.** The two CNT•Nic96<sup>R1</sup> complexes are colored in blue (CNT) and magenta (Nic96<sup>R1</sup>) and yellow (CNT) and red (Nic96<sup>R1</sup>), respectively. The two sAb-158 molecules are colored in gray.

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